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UNIVERSITY OF ALBERTA

PEPTIDE CHEMISTRY ANSWERS QUESTIONS IN MUSCLE REGULATION

by

JENNIFER E. VAN EYK

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1991

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TITLE OF THESIS: PEPTIDE CHEMISTRY ANSWERS QUESTIONS IN MUSCLE REGULATION

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: FALL 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**Peptide Chemistry Answers Questions in Muscle Regulation**" submitted by Jennifer E. Van Eyk in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Zenon Grabarek

Date: August 30, 1991

For Rob and Amy

ABSTRACT

The purpose of this thesis is to expand our knowledge about the molecular mechanism involved in the regulation of muscle contraction. Peptide chemistry is used to answer questions in muscle regulation by identifying regions of proteins that constitute the components of the chemical switch which control the Ca^{2+}-dependent interactions between the muscle proteins: troponin C, troponin I, actin and myosin. Synthetic peptides of TnI residues 104-115 and actin residues 1-28 were extensively used to probe the interactions between the various components of the switch.

Synthetic peptide analogs were used as probes in determining the exact amino acid residues or regions involved in the interaction with their target proteins and the conformational changes associated with these interactions. It was previously known that the synthetic TnI peptide 104-115 mimics its native protein, TnI. The TnI peptide inhibits the S1 ATPase activity and binds to TnC causing the release of the ATPase inhibition. Analysis of glycine-substituted TnI peptide analogs showed that every residue of the TnI sequence is necessary for maximum inhibition of the ATPase activity and for binding to TnC. As well, synthetic TnI peptides were used to determine the relationship between Ca^{2+} binding and the transmission of the Ca^{2+} signal through the thin filament proteins. The skeletal and cardiac TnI peptides (the skeletal and cardiac TnI inhibitory peptides have identical amino acid sequences except at position 110) alters Ca^{2+} binding at the regulatory low affinity Ca^{2+} binding sites of TnC . On the other hand, the glycine-substituted analog (at position 110) primarily affects the high affinity Ca^{2+} binding sites of TnC. Hence, the single amino acid difference in the TnI inhibitory sequence could be partly responsible for the biological differences between skeletal and cardiac muscle.

The synthetic actin peptide 1-28 mimics its native protein, actin. The actin peptide activates the myosin subfragment 1 (S1) and heavy meromyosin (HMM) ATPase activities

in a manner similar to the activation observed at low concentrations of F-actin. In addition, actin peptide 1-28 interacts with TnI, in particular the inhibitory region (residues 104-115). Importantly, the actin peptide 1-28 interacts with TnI (when part of the Tn complex) only in the absence of saturating Ca^{2+}. This indicates that the actin-TnI interaction is sensitive to the binding of Ca^{2+} to TnC.

Analysis of synthetic truncation analogs of the N-terminal region of actin showed that the N- and C-terminal regions are critical for activation of the ATPase activity while the N-terminal region is important for the interaction with TnI. In addition, the broader question dealing with the relationship between structure and function was addressed. The NMR derived structure of the TnI and actin peptide bound to its target protein(s) has been correlated with the information obtained from the bioassays using the synthetic peptide analogs studies.

A model for muscle regulation is proposed based on the results obtained using the synthetic TnI and actin peptides. The corresponding regions of TnI and actin are able to flip-flop between their two target proteins. The interactions which dominate depend on whether Ca^{2+} is bound to the regulatory low affinity Ca^{2+} binding sites of TnC. This, in turn, dictates whether or not the muscle fibre is relaxed or contracted. Clearly, the TnI inhibitory region and the N-terminal region of actin are two important components of the switch that controls muscle regulation.

ACKNOWLEDGMENTS

First and foremost, I thank my husband Rob for his encouragement and love which got me through the ups and downs of the last few years. Secondly, I would like to thank Bob Hodges, my supervisor who allowed independent thought and action while still being available for help. This is a reflection of his vast knowledge and expertise in a wide range of areas and his ability to get the best out of the people who work for him.

I must acknowledge the people, past and present associated with the Hodges laboratory, who have all contributed to make a wonderful working environment. In particular, I thank Colin Mant and Lorne Burke whose expertise coupled with their willingness to help with the practical aspects of HPLC saved me incredible amounts of time; Paul Semchuk for synthesizing the majority of the actin peptides; and Paul Cachia who originally introduced me to the field of muscle regulation. Finally, I feel I would never have finished this thesis had it not been for Morris Aarbo who spent the last 5 years purifying an enormous number of peptides.

A special thanks to the secretaries, Dawn Lockwood and Vicki Luxton for typing and retyping many manuscripts, and especially to Dawn who helped to prepare this thesis.

The group of professors who make up the MRC Group in Protein Structure and Function; Dr. Smillie, Dr. Kay, Dr. Sykes and Dr. James all contributed in their own ways to the work presented in this thesis. The cooperation between the various laboratories is a reflection of their love of science and their desire to understand all aspects of muscle regulation. I would like to acknowledge the people who work in these laboratories, especially Kim Oikawa for collecting CD and fluorescence data; Mike Nattriss for doing hundreds of amino acid analyses; Pat Campbell and Frank Soennichisen for sharing the NMR determined structures of the TnI and actin peptides prior to publication and finally Natalie Strynadka for sharing her excitement about science. As well, Prof. Dr. J.C. Rüegg at the Universat Heidelberg for investigating the effect of synthetic peptides on the force development in skinned muscle fibres.

A special thank you to Peggy Graham and Beth Monroe-Hill for editing this thesis and looking after Amy during the final throws of this work. In addition, I would especially like to acknowledge my family and friends who helped me keep life in perspective and who always assumed I would graduate.

TABLE OF CONTENTS

Page

CHAPTER 1: Introduction 1

MODELS FOR MUSCLE REGULATION 9

INTERACTION OF TnI AND ACTIN WITH OTHER MUSCLE PROTEINS 12

PURPOSE OF THIS STUDY 16

REFERENCES 20

CHAPTER 2: Materials and Methods 26

PEPTIDE SYNTHESIS 26

PEPTIDE PURIFICATION 28

PROTEIN PURIFICATION 29

SODIUM DODECYL SULFATE UREA - POLYACRYLAMIDE GEL ELECTROPHORESIS 32

ATPase ASSAYS 32

Continuous Titration Assay 34

AFFINITY CHROMATOGRAPHY OF PEPTIDES 34

Conventional Affinity Chromatography 34

High Performance Affinity Chromatography (HPAC) 35

SIZE-EXCLUSION CHROMATOGRAPHY 36

CENTRIFUGATION STUDIES 38

SKINNED MUSCLE FIBRE ASSAYS 38

CIRCULAR DICHROISM 39

FLUORESCENCE SPECTROSCOPY 40

DETERMINATION OF Ca^{2+} BINDING CONSTANTS 41

REFERENCES 42

CHAPTER 3: High-Performance Affinity Chromatography (HPAC) of Peptides and Proteins

INTRODUCTION 44

GENERAL PRINCIPLES OF HPAC 45

ADVANTAGES OF HPAC OVER CONVENTIONAL AFFINITY CHROMATOGRAPHY 45

CHOICE OF LIGAND AND LIGAND ATTACHMENT 48

RUN CONDITIONS IN HPAC 53

Choice of Buffer 53

Sample Recovery Techniques 53

Flow Rates 59

CARE AND MAINTENANCE OF COLUMNS 63

REFERENCES 64

CHAPTER 4: Calmodulin and Troponin C: Affinity Chromatographic Study of Divalent Cation Requirements for Troponin I Inhibitory Peptide (Residues 104-115), Mastoparan and Fluphenazine Binding

INTRODUCTION 65

RESULTS AND DISCUSSION 67

RECENT DEVELOPMENTS 75

REFERENCES 78

CHAPTER 5: The Biological Importance of Each Amino Acid Residue of the Troponin I Inhibitory Sequence 104-115 in the Interaction with Troponin C and Tropomyosin-Actin

INTRODUCTION 82

RESULTS 82

Inhibitory Action of the TnI 104-115 Synthetic Analogs on the Acto-S1 ATPase 82

TnC High Performance Affinity Chromatography of Synthetic TnI Analogs in the Presence of Mg^{2+} or Ca^{2+} 88

DISCUSSION 92

RECENT DEVELOPMENTS 97

REFERENCES 101

CHAPTER 6: Inhibition of TnI-TnC Interaction and Contraction of Skinned Muscle Fibres by the Synthetic TnI Peptide (104-115)

INTRODUCTION 103

RESULTS 104

Circular Dichroism Spectra Analysis of the TnC and TnI Peptide Interaction 104

Circular Dichroism Spectra Analysis of the TnI-TnC and TnI-Peptide 104-115 Interaction 105

The Interaction of Skinned Skeletal Muscle Fibres with TnI Peptide 104-115 109

The Interaction of Cardiac Muscle Fibres with the TnI Peptide 104-115 112

The Interaction of Skeletal and Cardiac Skinned Muscle Fibres with Mastoparan 114

DISCUSSION 115

RECENT DEVELOPMENTS 117

REFERENCES 120

CHAPTER 7: A Comparative Study of the Interactions of Synthetic Peptides of the Skeletal and Cardiac TnI Inhibitory Region With Skeletal and Cardiac TnC

INTRODUCTION 122

RESULTS 123

Secondary Structure of the TnI peptides in solution 123

The Effects of the TnI peptides on the Secondary Structure of Skeletal and Cardiac TnC 126

Ca^{2+} Titrations of the TnI peptide-TnC Complexes 129

DISCUSSION 134

REFERENCES 139

CHAPTER 8: A Synthetic Peptide of the N-terminus of Actin Interacts With Myosin

INTRODUCTION 142

RESULTS AND DISCUSSION 142

Actin Peptide 1-28 Interaction with S1 142

Actin Peptide Interaction with Acto-S1 and HMM 148

Competition Between the Actin Peptide and F-actin 153

CONCLUSION 153

REFERENCES 155

CHAPTER 9: Synthetic Peptide Studies on the Interaction of the N-terminal Region of Actin with Myosin Subfragment 1 (S1) and Heavy Meromyosin (HMM)

INTRODUCTION 157

RESULTS 157

The Effect of Actin Analogs on the S1 ATPase Activity 159

The Effect of Actin Analogs on the Acto-S1 ATPase Activity 163

The Effect of Actin Analogs on the HMM ATPase Activity 166

DISCUSSION 168

REFERENCES 172

CHAPTER 10: Synthetic Peptide Studies on the Interaction of the N-terminal Region of Actin with Troponin

INTRODUCTION 175

RESULTS AND DISCUSSION 175

Interaction Between Actin 1-28 and Tn 175

Interaction Between Actin 1-28 and TnI 177

RECENT DEVELOPMENTS 179

REFERENCES 183

CHAPTER 11: Discussion

EXPANDED MODEL OF MUSCLE REGULATION 184

MOLECULAR EVENTS THAT CONTROL MUSCLE REGULATION 187

Transmission of the Ca^{2+} signal via TnI 187

Transmission of the Ca^{2+} signal via actin to S1 190

FUTURE INVESTIGATIONS 192

REFERENCES 193

LIST OF TABLES

		Page
Table 1	Properties of various muscle proteins	3
Table 2	Sites of interaction between muscle proteins	5
Table 3	Examples of underivatized HPAC columns	50
Table 4	Effect of run conditions on chromatographic parameters - peptide elution from protein HPAC column	61
Table 5	Structure of ligands bound to the affinity column	66
Table 6	Binding of CaM and TnC to affinity columns	70
Table 7	Amino acid sequence of the TnI inhibitory peptide (104-115) and glycine analogs	83
Table 8	Evaluation of acto-S1 ATPase inhibition by TnI, TnI inhibitory peptide, and single glycine substituted TnI inhibitory analog	87
Table 9	Retention time of the TnI analogs on a TnC affinity column	91
Table 10	Amino acid sequence of TnI peptide and mastoparan	103
Table 11	Structural changes induced by Ca^{2+} and TnI peptide (104-115) in TnC and IC complex	107
Table 12	Effect of TnI peptide (104-115) and mastoparan on the Ca^{2+} activated force in skinned muscle fibres	113
Table 13	Amino acid sequence of skeletal and cardiac TnI inhibitory peptides and Gly 110 analog	124
Table 14	Number of additional α-helical residues induced in skeletal and cardiac TnC by the TnI inhibitory peptides and Ca^{2+}	127
Table 15	Effect of TnI peptides on the pCa values for low and high affinity Ca^{2+} binding sites of skeletal and cardiac TnC	133
Table 16	Determination of the binding of actin peptide 1-28 to the actin and acto-S1 complex	151
Table 17	Apparent binding constant and maximum activation of actin peptide 1-28 for S1 and HMM	152
Table 18	Amino acid sequence of N-terminus of actin, synthetic actin peptide 1-28 and truncated actin analogs	158

Table 19 The analysis of the S1-ATPase activity by the actin peptides 161

Table 20 The analysis of ATPase activation by actin peptides 165

Table 21 Retention of the actin analogs during TnI affinity chromatography 178

LIST OF FIGURES

		Page
Figure 1	A model for the molecular arrangement of the thin filament proteins	2
Figure 2	Schematic diagram of myosin	7
Figure 3	Simplistic scheme for the biochemical and kinetic steps involved in ATP hydrolysis during muscle contraction	8
Figure 4	Structure of turkey troponin C	13
Figure 5	Structure of G-actin derived from the actin:DNAase 1 complex	17
Figure 6	Schematic representation of affinity chromatography	46
Figure 7	The chemistry of ligand derivatization to epoxy-silica matrix	52
Figure 8	Comparison of retention times of single and multiple glycine-substituted troponin I peptide (104-115) analogs on a troponin C-HPAC column	56
Figure 9	Comparison of step gradient elution and linear gradient elution of troponin I peptide from a troponin C-HPAC column	58
Figure 10	The effect of flow-rate on the elution of troponin I peptide from a troponin C-HPAC column	60
Figure 11	The effect of Mg^{2+} or Ca^{2+} on the binding of TnC or CaM to a mastoparan-Sepharose affinity column	73
Figure 12	Inhibition of acto-S1-tropomyosin ATPase activity by TnI, TnI inhibitory peptide (104-115) and single glycine-substituted analogs of this region	84
Figure 13	Reciprocal plots of the TM-acto-S1-tropomyosin inhibition by TnI, TnI inhibitory peptide, analogs Gly 105, Gly 107, Gly 110 and Gly 114 versus inhibitor concentration	86
Figure 14	Comparison of retention times of single and multiple glycine-substituted TnI analogs on a HPAC-TnC affinity column in the presence of Ca^{2+}	89
Figure 15	Amino acid residues of the TnI inhibitory region (104-115) proposed to be involved in the interaction with TnC and TM-actin	94

Figure 16 Stereo-diagram of TnI inhibitory peptide (104-115) bound to Ca^{2+} saturated TnC 99

Figure 17 Effect of Ca^{2+} and TnI peptide (104-115) on circular dichroism spectra at 222 nm of TnC and IC complex 106

Figure 18 Inhibition of contraction of skinned psoas fibres by TnI peptide (104-115) 109

Figure 19 Dose dependence of TnI peptide inhibition in skinned psoas fibres 110

Figure 20 The TnI peptide decreases Ca^{2+} sensitivity in skinned psoas fibres 111

Figure 21 Dose dependence of TnI peptide inhibition in skinned pig cardiac fibres 113

Figure 22 Ca^{2+}-force relationship in skinned pig cardiac fibres before addition of the peptide and in its presence and after washing out the peptide..... 114

Figure 23 Inhibition of contraction of skinned psoas fibres by mastoparan 115

Figure 24 Comparison of fluorescence and force development in the absence and presence of TnI peptide (104-115) in psoas skinned muscle fibres at two Ca^{2+}concentrations 118

Figure 25 Circular dichroism spectra of TnI peptides in the absence and presence of 50% TFE 125

Figure 26 Effect of inhibitory peptides on Ca^{2+} binding to skeletal and cardiac TnC monitored by circular dichroism 131

Figure 27 Effect of inhibitory peptides on Ca^{2+} binding to skeletal and cardiac TnC monitored by Tyr fluorescence..... 132

Figure 28 Actin peptide 1-28 and S1 interaction determined by size-exclusion chromatography 144

Figure 29 Interaction between actin peptide 1-28 and S1 in the absence and presence of ATP 146

Figure 30 The effect of F-actin and actin peptide 1-28 on the acto-S1 and HMM ATPase activities..... 149

Figure 31 Analysis of the acto-S1 and HMM ATPase activities by the actin peptide 1-28 152

Figure 32 Competition results of F-actin and actin peptide in the S1 ATPase assay..... 154

Figure 33 The effect on S1 ATPase activity by C-terminal, N-terminal and C- and N-terminal truncated actin 1-28 analogs 160

Figure 34 The effect on acto-S1 ATPase activity by C-terminal, N-terminal and C- and N-terminal truncated actin 1-28 analogs 164

Figure 35 The effect on HMM ATPase activity by C-terminal, N-terminal and C- and N-terminal truncated actin 1-28 analogs 167

Figure 36 Actin peptide 1-28 and Tn interaction determined by size-exclusion chromatography..... 176

Figure 37 Elution profile of actin peptide 1-28 from skeletal TnI HPAC column 177

Figure 38 Competition between TnI and TnI peptide (104-115) or mastoparan for actin peptide (1-28)..... 180

Figure 39 Schematic showing the dominate protein interactions involved in the regulation of muscle contraction 186

Figure 40 Cross section of skeletal muscle thin and thick filaments and possible changes in the protein-protein interactions upon Ca^{2+} binding 188

ABBREVIATIONS USED:

A1/A2 light chains, small molecular weight polypeptides associated with myosin S1 head which can be remove from S1 heavy chain under alkali conditions.

acto-TnI, actin and troponin I complex

acto-S1, actin and myosin subfragment 1 complex

Ala (A), alanine

Arg (R), arginine

Asn (N), asparagine

Asp (D), aspartic acid

Boc, N^{α}-tert-butyloxycarbonyl

2-ClZ, 2-chlorobenzyloxycarbonyl

CD, circular dichroism

CNBr, cyanogan bromide

cIp, cardiac TnI peptide (137-148)

cTnC, cardiac troponin C

Cys (C), cysteine

Danz, 5-dimethylaminonaphthalene-2-sulfonyl aziridine

DCC, dicyclohexylcarbodiimide

DIEA, diisopropylethylamine

DMF, dimethyl formamide

DTT, dithiothreitol

DTNB light chains, small molecular weight polypeptides associated with myosin S1 head which can be removed from the S1 heavy chain with treatment of DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]

EDEANS, N-(5-sulfo-1-naphthyl)ethylenediamine

EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide

EDT, 1,2-ethanedithiol

EDTA, ethylene-diaminetetra-acetic acid

EGTA, ethylene glycol bis(β-amino ethyl ether) N,N,N',N'-tetraacetic acid

F, fluorescence

Glu (E), glutamic acid

Gly (G), glycine

Gly 110, denotes the Gly substituted analog at position 110 of skeletal TnI peptide (104-115) which is equivalent to Gly 143 in cardiac TnI peptide sequence (137-148)

HEPES, N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid

HF, hydrofluoric acid (anhydrous)

HMM, heavy meromyosin

HOBT, 1-hydroxybenzotriazole

HPAC, high performance affinity chromatography

HPLC, high performance liquid chromatography

HR, number of helical residues

IC, troponin I and troponin C complex

Ile (I), ileucine

Leu (L), leucine

Lys (K), lysine

βME, β-mercaptoethanol

Met (M), methionine

MOPS, 3-(4-Morpholino)propane sulfonic acid

NaN_3, sodium azide

NOE, nuclear Overhauser enhancement effect

NHS, N-hydroxysuccinimide

NMR, nuclear magnetic resonance

NS, synthetic peptide comprising the minimum sequence required for maximum biological activity (TnI peptide 104-115 and actin 1-28)

Phe (F), phenylalanine

Pi, inorganic phosphate

PMSF, phenylmethylsulfonyl fluoride

Pro (P), proline

S1, myosin subfragment 1

sIp, skeletal TnI peptide (104-115)

sTnC, skeletal troponin C

TFA, trifluoroacetic acid

TFE, trifluoroethanol

t_g or t_d, gradient delay time

Thr (T), threonine

TM, tropomyosin

Tn, troponin

TnC, troponin C

TnI, troponin I

TnI inhibitory peptide (104-115), a synthetic peptide of the skeletal TnI residues 104-115 (also refered to as sIp, NS and TnI peptide 104-115)

TnT, troponin T

Tos, 4-toluenesulfonyl

Tris, tris-(hydroxymethyl) amino methane

trNOESY, transferred nuclear Overhauser enhancement effect spectroscopy

Tyr (Y), tyrosine

Val (V), valine

CHAPTER 1

INTRODUCTION

Muscle contraction is initiated by nerve stimulation which causes an increase in the intracellular free Ca^{2+} concentration from lower than 10^{-7} M in the resting cell to about 10^{-5} M. At this concentration, Ca^{2+} binds to the regulatory complex tropomyosin-troponin (TM-Tn) present in the thin filament of the myofibril. The thin filament is composed of F-actin, tropomyosin and Tn (Fig 1). Table 1 lists properties of several proteins which are present in the thin filament. Actin is a highly conserved protein (Vandekerckhove and Weber, 1978) which can exist as a globular molecule (G-actin) or polymerize to form a filamentous strand (F-actin). In muscle, F-actin consists of two strands coiled about one and other to form long filaments. The two α-helical polypeptide chains of TM which are arranged in a coiled-coil lie head to tail in the two grooves formed by the F-actin filament. One TM spans approximately seven actin molecules and binds a single Tn complex. Tn is positioned at regular intervals (385 Å) on the thin filament such that there is a mole ratio of actin to TM to Tn of 7:1:1. Tn is a complex of three proteins consisting of TnC, a Ca^{2+} binding protein; TnI, the inhibitory protein; and TnT, which binds TM. Tn is a group of highly conserved molecules. The structure of avian TnC has been determined by X-ray crystallography and is discussed in detail in a later section. Skeletal TnC binds four Ca^{2+} ions, two at the high affinity Ca^{2+} binding sites (III and IV) and two at the low affinity sites (I and II). On the other hand, cardiac TnC binds three Ca^{2+} ions since the regulatory Ca^{2+} binding site I is non-functional. The three proteins which comprise Tn interact with one another and with TM-actin (Table 2). The changes in interactions between these various proteins provide the mechanism for Ca^{2+}-sensitive regulation of muscle contraction (a basic description of muscle and its regulation can be found in biochemistry textbooks such as Voet and Voet, 1990 and Stryer, 1988).

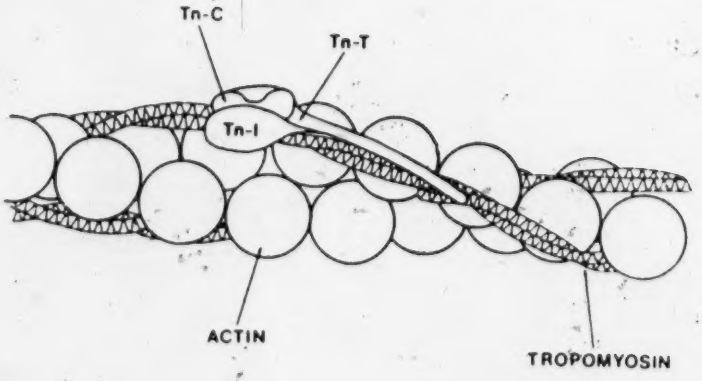

Figure 1. A model for the molecular arrangement of the thin filament proteins. Two strands of actin molecules (open circle) form the core of the structure. Tropomyosin (TM) molecules are illustrated as coiled-coils which lie in the grooves of the actin filament. The troponin complex (TnI, TnT, and TnC) interacts with the C-terminus of the TM molecules. Tn-TM form the basic regulatory unit of the thin filament (Heeley *et al.*, 1987).

Table 1. Properties of various muscle proteins

Protein (or fragment)	Molecular Weight	Number of Amino Acids	Characteristic	Reference
Myosin	470,000		ATPase activity	Lowey & Cohen, 1961
S1	95,000		ATPase activity	Weeds & Taylor, 1975
HMM	365,000		ATPase activity	Weeds & Pope, 1977
Actin	42,000	375	activates ATPase	Elzinga *et al.*, 1973
α-TM	33,000	284	binds actin &TnT	Stone *et al.*, 1974
cardiac TnC	18,416	161	binds 3 Ca^{2+}	Van Eerd & Takahashi, 1976
skeletal TnC	17,846	159	binds 4 Ca^{2+}	Collins *et al.*, 1977
skeletal TnI	20,864	178	inhibits ATPase	Wilkinson & Grands, 1975,1978
skeletal TnT	30,503	259	binds TM	Pearlstone *et al.*, 1976

At resting levels of Ca^{2+}, the high affinity Ca^{2+} binding sites of TnC are filled with Mg^{2+} or Ca^{2+}. After nerve stimulation and the subsequent increase in the concentration of free Ca^{2+}, the low affinity site(s) bind Ca^{2+}. The signal that initiates muscle contraction is Ca^{2+} binding to the low affinity Ca^{2+} binding sites which causes conformational changes in TnC. These Ca^{2+}-dependent changes are transmitted to the other muscle proteins of the thin filament and ultimately alter the spatial relationship between the thin and thick filaments. The thick filament is comprised of myosin which is an enzyme capable of hydrolyzing ATP (ATPase activity). Myosin is composed of two heavy chains and four associated light chains. The carboxy terminal regions of the two heavy chains form a two-stranded α-helical coiled-coil, while the two amino terminal regions fold into two globular heads. Each globular head binds actin (thin filament) and can hydrolyze ATP. When actin binds to the myosin head the ATP hydrolysis rate is increased due to an increase in the rate of release of the products from the ATP hydrolysis. Myosin can be specifically cleaved at several sites (as indicated in Fig. 2) to yield a single-headed myosin subfragment 1 (S1) or a double-headed heavy meromyosin fragment (HMM). The two DTNB light chains which are not essential for ATPase activity are destroyed during the cleavage process used to prepare S1 and HMM. The other two light chains known as A1 (MW 21,000) and A2 (MW 16,200) are essential for ATPase activity and remain associated with the myosin fragments. Both myosin fragments, S1 and HMM, retain the ability to bind actin and hydrolyze ATP.

The Ca^{2+} signal alters the acto-myosin interaction such that there is an increase in the number of crossbridge attachments between the thick and thin filaments. Fig. 3 is a schematic of the mechanical and kinetic steps involved in muscle contraction. During contraction, myosin forms a crossbridge with F-actin. This interaction results in an increase in the myosin ATPase activity. This provides the energy required for the sliding between adjacent actin and myosin filaments (the power stroke), thus producing muscle

Table 2. Sites of interaction between muscle proteins

Protein Bound	Putative Binding Region (amino acid residues)					
	TnC	TnI	TnT	TM	Actin	Myosin (S1)
TnC		1-47[2,a] 104-115[a,b,c]	176-230[2,e] 159-259[2,d]			
TnI	50-60[2,f,g] 89-100[2,f,g,h] 126-136[g]		152-209[i]		1-44[k] 1-12[j] 1-28[q]	
TnT	1-100[2,g,l] 121-159[f,l]	40-78[m]		190-284[7,aa,bb,cc]		
α-TM		104-115[a,b,c]	71-151[bb] 197-259[2,y]		95[6,v], 61[w] 70-86[z] 340-365[z]	237-335[5,x]
Actin		96-116[a,c,k] 104-115[b]				A1 light chain[p,n] 702-708[3,r] 20 and 50 kDa junction[4,s,t,u]
Myosin (S1)					1-28[q] 1-12[n] 61[w] 40-113[o] 361-364[p]	

1 In some cases the actual binding site lies within this stretch of residues.
2 Interaction known to be Ca^{2+}-dependent.
3 Known as the S-site.
4 Known as the J-site.
5 Based on modification of Lys.
6 Based on modification of Arg.
7 Based on modification of Cys.

a Syska *et al.*, 1976.
b Talbot and Hodges, 1979,1981a,b.
c Nozaki *et al.*, 1980.
d Pearlstone and Smillie, 1978.
e Ohtsuki, 1979.
f Leavis *et al.*, 1978.
g Grabarek *et al.*, 1981.
h Weeks and Perry, 1978.
i Pearlstone and Smillie, 1980.
j Grabarek and Gergely, 1987.
k Levine et *al.*, 1988.
l Ohara *et al.*, 1980.
m Hitchcock *et al.*,1981.
n Sutoh, 1982.
o Bertrand *et al.*, 1988.
p Trayer *et al.*, 1987.
q Van Eyk and Hodges,1991
r Suzuki *et al.*, 1987.
s Yamamoto and Sekine, 1979.
t Mornet *et al.*, 1979
u Sutoh, 1983.
v Johnson and Blazyk, 1978
w Barden and Phillips, 1990.
x Szilagi and Lu, 1982.
y Pearlstone and Smillie, 1981.
z Johnson and Stockmal, 1982.
aa Mak and Smillie, 1979.
bb Pato *et al.*, 1981.
cc Chong and Hodges, 1982.

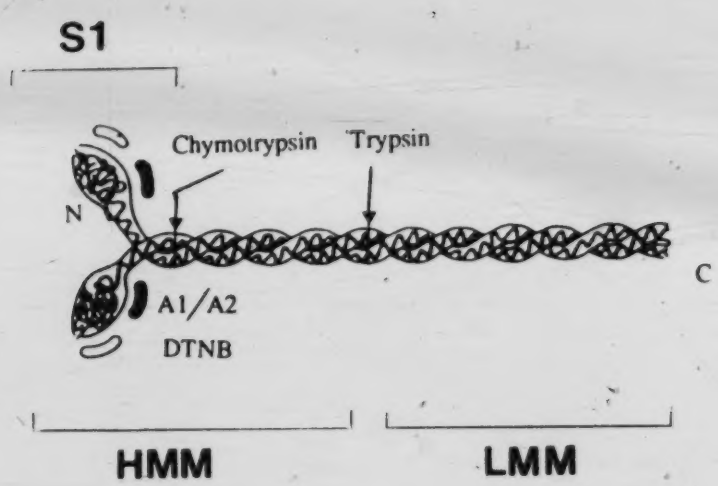

Figure 2. Schematic diagram of myosin. The molecule is composed of six polypeptide chains: two heavy chains and two pairs of light chains. S1, HMM and LMM are fragments generated by limited proteolysis at the points indicated.

contraction. The power stroke is the mechanical mechanism by which the myosin head tilts from 45° to 90° causing the filaments to push past one another. The crossbridges appear not to be statically attached to the thin filament but rather to be in a rapid equilibrium between attached and detached states. Thus muscle tension is a function of the sum of the number of crossbridges at any point in time (Schoenberg *et al.* 1984). Once nerve stimulation is over, the free Ca^{2+} concentration is lowered to the resting level. This process reverses the interaction between the various proteins so that the ATPase activity is inhibited and the

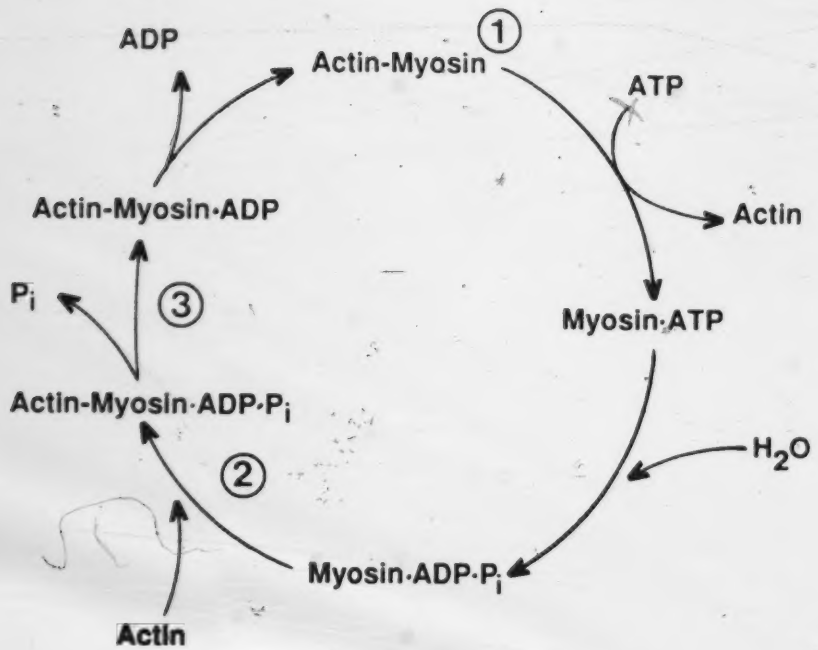

Figure 3: Simplistic scheme for the biochemical and kinetic steps involved in the ATP hydrolysis during muscle contraction.

[1] Other complexes can exist such as myosin, myosin-ADP-Pi, myosin-ADP and acto-myosin-ATP, depending on the various binding constants. However, myosin hydrolysis of ATP occurs at a low rate compared to actin-activated ATPase activity.

[2] Crossbridge formation occurs upon actin (TM-Tn-actin) interaction with myosin.

[3] This step is proposed to be regulated by Tn. In the absence of Ca^{2+}, Tn-TM blocks the release of Pi, while in the presence of Ca^{2+}, regulated actin enhances release of Pi. This results in a quicker cycling of the power stroke with the movement of the myosin with respect to the thin filament. In the allosteric model, this transition from the weak (actin-myosin-ADP-Pi) to the strong (actin-myosin-ADP and actin-myosin) binding state constitutes the 90^{o} to 45^{o} movement of the myosin head (power stroke).

muscle relaxes (see review articles: Leavis and Gergely, 1984; and A.S. Zot and Potter, 1987). There are several unresolved questions concerning control of muscle contraction: what is the exact molecular mechanism involved in the interaction between the thin filament proteins in the regulation of acto-myosin interaction and, what is the relationship between the biochemical steps (at a molecular level) in the acto-myosin interaction and the mechanical properties (power stroke) of the muscle fibres.

MODELS FOR MUSCLE REGULATION

The present model of muscle regulation which incorporates the previous steric blocking and allosteric models proposes that in the relaxed state, the positions of TnI and TM are such that myosin ATPase is inhibited (see review article El-Saleh *et al.*, 1986). In the presence of Ca^{2+}, the movement of Tn-TM on the actin filament activates the ATPase activity due to increased interaction between actin and myosin. In the early 1970's, the steric blocking model was proposed based on the 3D reconstructions from electron micrographs of negatively stained actin filaments decorated with S1 and on X-ray diffraction studies showing a shift in the position of TM on the thin filament (Huxley, 1972; Haselgrove 1972; Parry and Squire 1973; Wakabyashi *et al.*, 1975; and Potter and Gergely, 1974). The steric blocking model predicted that in the 'off' position (muscle relaxation) TM was situated close to the attachment site for myosin and actin, and therefore physically was blocking the interaction between actin and myosin. When Ca^{2+} bound to Tn (muscle contraction), TM would roll in the actin groove ('on' position) and out of the way promoting the acto-myosin interaction. Regulation of the position of TM occurs via TnI, which, in the absence of Ca^{2+}, would be bound to actin-TM in such a way as to block acto-TM interaction with myosin, while in the presence of Ca^{2+}, the TnI-TM-actin interaction would be broken and TnI would interact with TnC.

The allosteric model, first proposed by Eisenberg *et al.* (1980), was an attempt to link the biochemical process with the mechanical steps involved in the power stroke. In this model, the actin and myosin crossbridges could be in any of four states -- two where the thin filament was not bound to myosin and two where the thin filament was bound. In order to model the mechanically significant kinetic steps in the crossbridge cycle, the two bound states were assumed to have very different mechanical properties from one another (Fig. 3). The initial crossbridge attachment between the actin and myosin (bound state #1) is weak (A-M-ADP-Pi). Upon phosphate release (bound state #2) there is a transition to a strongly bound state (A-M-ADP). The transition from weak to strong binding states of acto-myosin constitutes the 90° to 45° movement of the myosin head, in other words, the power stroke. An expansion of the allosteric model to include a mechanism for regulation of muscle contraction (Chalovich *et al.*, 1983) proposes that regulated actin (actin-TM-Tn) in the presence of Ca^{2+} bound either myosin-ATP or myosin-ADP-Pi weakly at 90°(beginning of crossbridge cycle). At the end of the power stroke, regulated actin interacts strongly with myosin or myosin-ADP at 45°. The transition of the myosin head between 90° and 45° was assumed to occur rapidly and was associated with release of Pi. As Ca^{2+} was removed, TM assumed a different position on the thin filament (a position where it is in the weak binding state). This resulted in the destabilization of the interaction between actin-TM-Tn and myosin-ATP or myosin-ADP. By some mechanism, TM inhibited (blocked) the movement of the myosin head to 45°, promoting muscle relaxation. TM did not necessarily physically block acto-myosin interaction (as predicted by the steric blocking model) but rather blocked a kinetic transition (the release of Pi from the nucleotide active site), possibly through conformational change in myosin and/or actin (Lehrer and Morris, 1982). The current thought is that TM actually occupies several positions on the thin filament which are under the control of S1 and Tn (Hill, 1983; Hill *et al.*, 1984). The position to which TM is shifted (pushed) when S1 binds depends on which nucleotide is

bound to S1 (since the particular nucleotide may affect the angle at which S1 attaches to actin) and on the number of moles of Ca^{2+} bound to TnC (affecting conformation of the thin filament). This suggests that there is a relationship between the number of crossbridge attachments (acto-myosin interaction) and Ca^{2+} binding to TnC. There is some evidence from previous work to support this idea. Bremel and Weber (1972) demonstrated that the presence of rigor crossbridges (strong interaction between myosin and actin in the absence of nucleotide) increases the Ca^{2+} affinity of Tn, suggesting crossbridge attachment affects the conformation of TnC. The interaction of TM-Tn with actin lowered the affinity of the regulatory sites of TnC (Zot *et al.*, 1983; H.G Zot and Potter, 1987), suggesting a relationship between actin and TnC conformation. Recently, by incorporating a fluorescence probe (Danz) labelled TnC into TnC-depleted muscle fibres, it was shown that there were different TnC conformations and Ca^{2+} affinities depending on whether the myofibril was in a weak or strong crossbridge state (Zot and Potter, 1989). These results suggest that conformational changes due to crossbridge formation between actin and myosin influence the structural changes that occur when Ca^{2+} binds to TnC and vise versa.

The nature of the conformational differences in the S1 head due to the presence of a particular nucleotide, the conformational change induced by actin binding (hence the position of TM-Tn on the actin filament), and changes induced by Ca^{2+} binding to TnC are unclear. To clarify our understanding of these conformational changes in the thick and thin filaments, the exact sites of interaction between the various muscle proteins must be identified both in the presence and absence of Ca^{2+}. This involves determining the minimum amino acid sequence of each protein required for binding and biological activity. Next, it is necessary to identify the exact amino acid side chains involved in the protein-protein interactions. This thesis deals with the biologically important interactions between

TnI and actin or TnC and the interactions between actin and TnI or myosin. Both TnI and actin are key proteins involved in the regulation of muscle contractions.

INTERACTION OF TnI AND ACTIN WITH OTHER MUSCLE PROTEINS

A number of studies employing proteolytic fragments of TnC have revealed 3 sites of interaction between TnC and TnI: C-helix (residues 50-60), E-helix (residues 89-100) and the G-helix (residues 126-136) (Fig 4, Table 2). The interaction of TnI with the G-helix is not Ca^{2+}-dependent (Grabarek *et al.*, 1981), while the interaction with the other two regions are Ca^{2+}-sensitive. The X-ray crystallographic-determined structure of TnC allows the visualization of the regions of TnC involved in Ca^{2+} and TnI binding (Fig.4, Herzberg and James, 1985; Sundralingam *et al.*, 1985). TnC consists of two distinct domains, the N-terminal domain containing the Ca^{2+} binding sites I and II (low affinity sites), and the C-terminal domain containing binding sites III and IV (high affinity sites). The two domains are separated by a long, nine-turn α-helix, producing an elongated, dumbbell-shaped molecule. The central linking helix (D/E linker) is composed of a fusion of the D and E helices which are the C- and N-terminal helices of the Ca^{2+} binding sites II and III, respectively. Conformational changes due to Ca^{2+} binding at either site II or III appear to be transmitted between the two domains of TnC perhaps via the D/E linker. Grabarek *et al.* (1986), have shown that the binding of Ca^{2+} to the low affinity sites perturbs the structure at Cys 98 (part of helix E) which is located near Ca^{2+} binding site III. It is important to note that, Cys 98 is located near the probable binding site of the TnI inhibitory region (residues 96-116). In addition, a TnC mutant which was labeled in the N-terminal domain demonstrated that the binding of cations to the high affinity sites alters the environment around position 57 (Wang *et al.*, 1990). However, there is some evidence that TnC does not have an elongated shape at physiological pH but a more compact structure

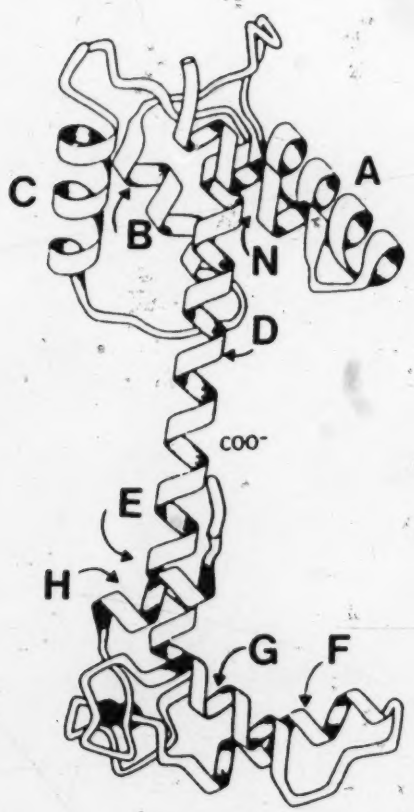

Figure 4. Structure of turkey troponin C. The crystallographic-determined structure of TnC by Herzberg and James, 1985 shown as a ribbon diagram. The N-terminal domain (top) is separated from the highly homologous C-terminal domain (bottom) by a long α-helix. The regulatory Ca^{2+} binding sites I and II located in the N-terminal domain are unoccupied in the crystal form. Sites III and IV in the C-terminal domain are filled with Ca^{2+} (solid circles). Helices involved in the Ca^{2+} binding sites are labelled A to H sequentially with the additional helix in the N-terminus of the molecule labelled as the N-helix.

(Heidorn and Trewhella, 1988; Hubbard *et. al.*, 1988 and Wang *et al.*, 1987, Fujisawa *et al.*, 1989). This means that the C- and N-terminal domains may bend, thus bringing the domains into close contact. The debate over the flexibility and function of the central helix (D/E) remains unsolved for the present time.

The crystal structure of TnC was obtained with Ca^{2+} binding sites I and II in the Ca^{2+} free state. This domain seems to be stabilized by the interactions between the various helices comprising the Ca^{2+} binding sites I and II and the N-helix (residues 3-13). It is interesting that there is no counterpart to the N-helix in the C-terminal domain. This suggests that the Ca^{2+} affinity for the low affinity sites may not only be a function of the residues within the Ca^{2+} coordinating loop but also a function of the tertiary folding of this domain. Herzberg *et al.*, (1986) predicted, based on computer modeling of the X-ray crystal structure of TnC, that residues in the C- and D-helix would become more exposed upon Ca^{2+} binding to the low affinity sites. NMR studies and studies modifying Cys and Lys (Hitchcock, 1981; Ingraham and Hodges, 1988; Fuchs *et al.*, 1989) indicate that the residues along the D-helix become more exposed to solvent upon Ca^{2+} binding, thus potentially opening a new binding site for TnI (possibly the N-terminal residues of TnI). Extensive modeling of TnC-binding molecules (TFP and mastoparan) shows one interaction site involves the hydrophobic pocket in the N-terminus of TnC, which becomes exposed when Ca^{2+} binds to sites I and II (Strynadka and James, 1988, 1990). The other site of TnI interaction is located in the C-terminal domain, in particular, at or near helix E of TnC. This latter site is probably the primary site of interaction with the TnI inhibitory region (discussed in later section, Chapters 4 and 7).

Studies on the proteolytic fragments of TnI identified two regions of TnI that could interact with TnC: residues 1-47 and 96-116 (Syska *et al.*, 1976, Table 2). It has been proposed that residues 1-47 interacts with TnC residues 50-60 while TnI residues 96-116 interacts with TnC residues 86-100. The TnI fragment comprising residues 96-116 is of

special interest because this fragment also binds to actin and inhibits the acto-myosin ATPase activity (Syska *et al.*, 1976). In fact, Talbot and Hodges (1979, 1981a,b), the first to use synthetic peptide of TnI, demonstrated that residues 104-115 comprised the minimum sequence required for inhibition of the ATPase activity. The synthetic TnI peptide, TnI 104-115, also mimics TnI by possessing TM specificity such that the addition of TM increases the inhibitory action of this peptide. As well, TnI 104-115 binds TnC resulting in the release of inhibition of the ATPase activity (Cachia *et al.*, 1983, 1986). Part of the work presented in this thesis (Chapters 4-7) continues to investigate the interaction of the TnI inhibitory peptide with TnC and actin in an attempt to broaden our knowledge of the role of TnI in the regulation of muscle contraction.

Less is known at the molecular level about the interaction between actin and its target proteins, TnI and myosin, than about the interaction involving TnI and TnC (Table 2). It has been only in the last few years that the regions involved in the acto-S1 and acto-TnI interaction have been determined. Immunological data, crosslinking, and NMR experiments have strongly indicated that the N-terminal region of actin contains the binding sites between actin and myosin. Crosslinking experiments have shown that the acidic residues of actin region (1-12) bound to the 20 and 50 kDa tryptic fragments of S1 (Sutoh, 1982, 1983). NMR studies have confirmed that residues in the amino terminus of actin were perturbed during the acto-S1 interaction (Moir and Levine, 1986, Moir *et al.*, 1987). Two independent laboratories produced antibodies specific to sequences within the N-terminal region of actin. Mejéan *et al.*, (1986, 1987) used two anti-protein antibodies with different specificities in the N-terminal region of actin 1-28. The antibody affected by sequence changes at positions 2 and 3 (skeletal *vs* cardiac actin) only partially inhibited the acto-S1 interaction (determined by ELISA assays). Their second antibody with the suggested epitope favoring the C-terminal region of residues 1-28 completely blocked the interaction of actin and S1. Miller *et al.* (1987) used an anti-peptide antibody to residues 1-7 to confirm that this region was not directly involved in the acto-S1 interaction (determined

by equilibrium dialysis). However, this anti-peptide antibody inhibited the S1-induced polymerization of G-actin by blocking the initial binding of these proteins (Das Gupta *et al.*, 1990). As well, the N-terminal region of actin has been implicated in the acto-TnI interaction, in particular, the TnI fragment 96-116 (Grabarek and Gergely, 1987; Levine *et al.*, 1988).

The recent actin structure determined by X-ray crystallography of the G-actin:DNAase I complex showed that regions within the N-terminal residues 1-32 of actin, in particular, residues 1-7 and 20-28 are located on the surface of the molecule (Fig. 5, Kabsch *et al.*, 1990). In the actin structure and the proposed model of the F-actin filament, the N-terminal residues are mainly exposed and not involved in either the DNAase I-actin or proposed actin-actin interaction (Holmes *et al.*, 1990). As well, the actin residues 1-7 had high temperature factors which indicate that this region is probably surface exposed and mobile. Another region with high temperature factors includes residues 95-100 which are part of another proposed site of interaction between actin and S1, residues 40-113 (Bertrand *et al.*, 1988). It would not be surprising if there were multiple interactions between S1 and actin that some of these interactions were sensitive to the Ca^{2+}-dependent changes in regulated actin and/or to nucleotide binding to S1. As with the structure of TnC determined by crystallography, the actin structure gives only a glimpse of what is occurring at the structural level. Until the structures of actin-S1 or actin-TnI are determined, the conformational changes due to protein-protein interactions must be determined by other biochemical methods. One such method is to determine the structure of synthetic peptides of the active sites of actin and TnI bound to their target proteins by modern NMR techniques.

PURPOSE OF THIS STUDY

The purpose of this thesis is to expand our knowledge about the exact molecular mechanisms involved in the regulation of muscle contraction. Peptide chemistry is used to

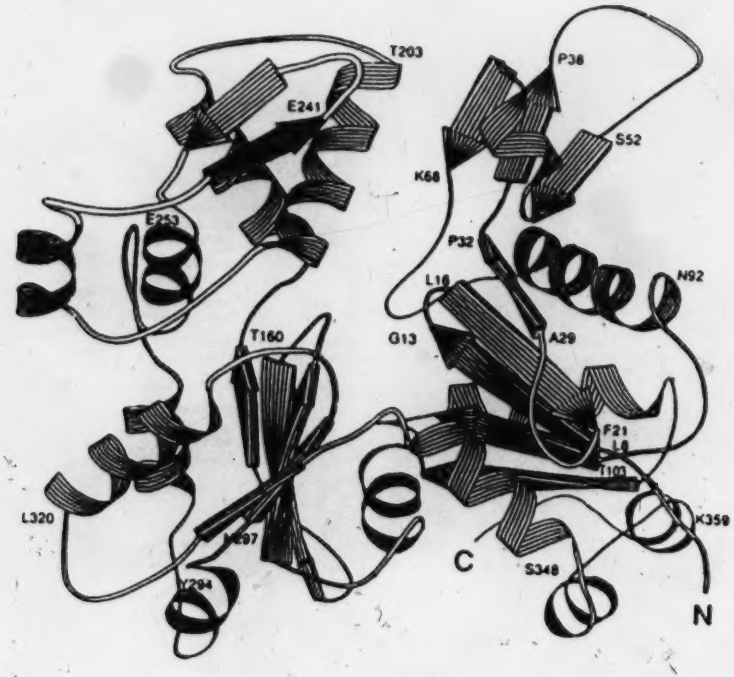

Figure 5. Structure of G-actin derived from the actin:DNAase 1 complex. The crystallographic determined structure of G-actin by Kabsch *et al.*, 1990 is shown as a ribbon diagram. The two domains (right and left) are separated by the Ca^{2+} and nucleotide binding clefts. Residues are labelled periodically throughout the molecule.

answer questions about muscle regulation by identifying regions of proteins that constitute the chemical switches which control Ca^{2+}-dependent interactions among muscle proteins. Synthetic peptides of biologically important regions of TnI and actin are used as probes in determining the exact residues or regions involved in these interactions between various muscle proteins. In addition, peptides can probe the conformational changes that occur upon interaction and the changes associated with the transmission of the Ca^{2+} signal. Finally, the broader question dealing with the relationship between structure and function is addressed by correlating information obtained from NMR derived structure of a synthetic peptide bound to its target proteins with the knowledge obtained from bioassays using synthetic peptide analogs.

The use of synthetic peptides to probe the molecular mechanism of muscle contraction has exploded in the past 3 to 5 years and includes synthetic peptides from almost all skeletal muscle proteins (TnI, TnC, S1 and actin). As stated earlier, the original studies using synthetic peptides to study interactions between muscle proteins were carried out by Talbot and Hodges (1979, 1981a,b). Research employing the synthetic TnI peptide 104-115 has been on-going over the last decade and includes work presented in this thesis. Synthetic peptide analogs of a Ca^{2+} binding site of TnC (loop or helix-loop-helix) have also been studied in an attempt to understand the variation in cation specificity and affinity and the effect of different residues on the conformation of the loop region (Gariépy *et al.*, 1982, 1983, 1985; Reid *et al.*, 1980, 1981; Marsden *et al.*, 1988, 1989, Shaw *et al.*, 1990). Synthetic peptides, having the amino acid sequence around the reactive thiol (SH1) of myosin (residues 702-708), bind F-actin (Suzuki *et al.*, 1987; Eto *et al.*, 1990, Suzuki *et al.*, 1990), while larger synthetic myosin peptides from this region, residues 690-725, contain two functionally distinct actin binding sites (Keane *et al.*, 1990). Finally, as presented in this thesis, synthetic peptides of the N-terminal region of actin can be used to probe the acto-myosin and acto-TnI interaction.

Synthetic peptides of TnI and actin have increased our understanding of the molecular events involved in the transmission of the Ca^{2+} signal through the thin filament proteins to the thick filament. This, in turn, alter the ATPase activity. The synthetic peptides have provided insight into the relationship between the Ca^{2+} specificity and affinity of TnC and the interaction between different proteins. Since HPAC is used extensively throughout this thesis, the conditions required for optimization of HPAC when synthetic peptides are employed as ligands or solute molecules are determined (Chapter 3). In Chapter 4, the cation requirements for the binding of three molecules: TnI synthetic peptide, mastoparan and fluphenazine to TnC and CaM are investigated. In other words, the three TnC and CaM-binding molecules are used to probe the subtle differences in theTnC and CaM binding interfaces due to binding of different cations. Chapter 5 examines the contribution of each amino acid of the TnI inhibitory peptide towards the interaction between this peptide and TM-actin and TnC. This work shows that one of the components of the Ca^{2+}-dependent switch in muscle regulation involves the TnI inhibitory region is flip-flopping between TM-actin and TnC . Chapter 6 deals with the ability of the TnI peptide to compete with TnI for TnC in solution and its effect on force development in skinned muscle fibres. The TnI peptide can inhibit IC interaction which may lead to Ca^{2+}-desensitization of the muscle fibre. Chapter 7 indicates that the TnI peptide can alter Ca^{2+} affinity at the regulatory low affinity binding sites of TnC (which are located in the N-terminal domain) even though a probable binding site of this peptide is located in the C-terminal domain. This result suggests that there maybe communication between the two domains of TnC. In addition, position 110 of the TnI peptide is critical, since the Gly 110 analog mainly affects the high affinity binding sites (located in the C-terminal domain), suggesting that the single amino acid difference in the TnI inhibitory sequence could be partly responsible for the biological differences between skeletal and cardiac muscle.

Another important discovery is that a synthetic peptide of the N-terminus of actin (residues 1-28) can activate S1 and HMM ATPase activities (Chapter 8). As shown in Chapter 9, the N- and C-terminal regions of actin 1-28 (residues 1-7 and 20-28) are essential for activation of the ATPase activity. This result correlates well with the X-ray crystallographic-determined structure of actin in which the central region of actin residues 1-28 is buried inside the protein, while residue 1-7 and 20-28 are located on the surface of the actin molecule. Chapter 10 shows that the synthetic peptide of actin 1-28 can bind Tn in the absence of Ca^{2+}. In particular, actin 1-28 interacts with TnI residues 104-115. Thus, actin 1-28 can interact with either myosin or TnI. This indicates that the N-terminus of actin is another component of the chemical switch involved in muscle regulation.

In this thesis, the TnI peptide (104-115) and the actin peptide (1-28) as well as a large number of synthetic analogs are used in a wide variety of biochemical methods to probe the mechanisms involved in the regulation of muscle. It is clear that the synthetic TnI and actin peptides comprise regions of their native protein that are important components of the chemical switch involved in the Ca^{2+}-sensitive regulation of muscle contraction.

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CHAPTER 2

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and solvents were reagent grade. Diisopropylethylamine (DIEA), dichloromethane, and trifluoroacetic acid were distilled prior to use. Picric acid was dissolved in dichloromethane and dried over magnesium sulfate. Acetonitrile (HPLC grade) was obtained from Fisher. Double-distilled water was purified by passage through a Milli-Q water purification system. Fluphenazine was a gift from E.R. Squibb and Sons Ltd., Montreal, Quebec. Boc-amino acids were purchased from Vega Biochemicals (Tucson, AZ), Bachem Fine Chemicals (Torrance, CA), Protein Research Foundation (Peptide Institute, Osaka, Japan), and Institut Armand Frappier (Quebec, Canada). Co-poly (styrene, 1% or 2% divinylbenzene)benzhydrylamine-HCl resin, 200-400 mesh (~0.8 meg. nitrogen/g) was purchased from U.C.B. Bioproducts, Belgium and Institut Armand Frappier, Quebec.

PEPTIDE SYNTHESIS

The peptides were synthesized on either a Beckman peptide synthesizer model 990 or an Applied Biosystems peptide synthesizer model 430A. Peptides were synthesized using the general procedure for solid-phase synthesis described by Parker and Hodges (1985) and Hodges *et al.* (1988).

All amino groups were protected at the α-amino position with the Boc-group and the following side-chain protecting groups were used: Arg(Tos) and Lys(2-ClZ). On the Beckman synthesizer all amino acids (except Arg) were double coupled as dicyclohexyl-carbodiimide (DCC) activated amino acids. The Boc-amino acid (3 eq.) in dichloromethane was added to the peptide resin followed by a solution of DCC (3.3 eq.) in dichloromethane (~4 ml). On the Applied Biosystem synthesizer, all amino acids were

double coupled as preformed symmetrical anhydrides (with the exception of Lys) first in DMF and then dichloromethane. The addition of Arg (on the Beckman synthesizer) or Lys (on the Applied Biosystems synthesizer) were coupled as 1-hydoxybenzotriazole (HOBT) active esters. The Boc-amino acid (5 eq.), HOBT (5.5 eq.) and DCC (7.5 eq.) in DMF (3 ml) were previously chilled to 4°C then mixed for 30 min at 25°C. The dicyclohexyl urea precipitate was removed by filtering through glasswool. The active ester was added to the peptide resin and mixed for 60 min. Difficult couplings were monitored using picric acid on the Beckman synthesizer or ninhydrin on either synthesizer. In the event that coupling efficiency was less than 99%, the amino acid was manually recoupled followed by blocking the unreacted groups by acetylation. The program used on the Beckman synthesizer for picrate monitoring consisted of: 0.1 M picric acid (from BDH) in dichloromethane, a dichloromethane wash to remove unbound picric acid followed by 5% DIEA in dichloromethane wash which was collected for the determination of the number of free amino groups. The collected DIEA/dichloromethane wash was diluted to a known volume with dichloromethane. The absorption of the solution was recorded at 362 nm. The total concentration of amino groups on the resin (μmole) was then calculated using a DIEA-picrate molar extinction of 15,100. When monitoring was performed after deprotection, a second deprotection cycle was carried out after the monitoring cycle was complete with a 5 min treatment of trifluoroacetic acid-dichloromethane (1:1) instead of 20 min. Monitoring by either picric acid or ninhydrin, were always carried out to determine the substitution of the first amino acid on the resin. It must be noted that ninhydrin monitoring is a destructive method since 1 to 2 mg of resin are removed from the batch resin each time analysis is preformed. On the other hand, picric acid monitoring is nondestructive since monitoring is done on the batch resin which is fully restored to its original state following analysis.

Boc-Gly(1-C^{14}) was used when incorporating a radioactive label into a synthetic peptide. The radioactive Boc-Gly was manually coupled as a preformed symmetric

anhydride. The coupling efficiency was between 50 to 80%. Following the radioactive coupling, cold Boc-Gly was coupled to its maximum. Finally, any unreacted amino groups were blocked by acetylation. Two synthesis of radioactive labelled actin peptide 1-28 (Gly 15 was labelled) were prepared with specific activities of either 290 or 1550 cpm/ nmole peptide.

On the Applied Biosystems synthesizer, Boc-groups were removed at each cycle with a short (80 sec) reaction with 33% or 50% TFA/dichloromethane (v/v), followed by a second longer reaction (18-25 min) with TFA/dichloromethane. Neutralizations were carried out using 10% DIEA/DMF (v/v). Following amino acid couplings, when necessary, any unreacted free amino groups present were acetylated with 25% acetic anhydride/dichloromethane (v/v) for 10-20 min. This procedure was also used to acetylate the α-amino group of the peptides following coupling of the final amino acid residue. The peptides, synthesized on either the Beckman or Applied Biosystem peptide synthesizers, were cleaved from the resin support by treatment with anhydrous hydrogen fluoride (20 ml/g resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at -4°C. The resins were then washed with ether, and the peptides were extracted with trifluoroacetic acid or 30% acetic acid. The trifluoroacetic acid or acetic acid extract was evaporated, and the peptide was redissolved in water and lyophilized.

PEPTIDE PURIFICATION

The crude TnI peptides and mastoparan used in Chapters 4 and 5 were purified on a HPLC system comprised of a Vista series 5000 liquid chromatograph (from Varian Associates, Walnut Creek, CA) and either a Varian CD 402 system, Kratos SF7296 detector, or an HP1040A detector, HP8513 computer, HP9121 disc drive, HP2225A inkjet printer, and HP7470A plotter (Hewlett-Packard, Palo Alto, CA). Initial separation was carried out on a weak cation-exchange column (SynChropak CM300, 250 x 4.1 mm I.D.) by employing a linear AB gradient, from 30% B to 50% B over 50 min (4 mM

KCl/min) at 1 ml/min. Buffer A consisted of 5 mM KH_2PO_4, pH 6.5 and buffer B was 5 mM KH_2PO_4 plus 1 M KCl, pH 6.5. The SynChropak CM 300 column is extremely efficient at separating highly basic peptides. Cachia *et al.* (1983) demonstrated that the column could separate two basic peptides containing an equivalent number of charged groups but differing in the position or type of charged residues. The peptides were subsequently desalted and further purified by reversed-phase chromatography on a SynChropak RP-P C_{18} column (250 x 4.1 mm I.D.) by employing a linear AB gradient (1% B/min) at 1 ml/min, where buffer A was 0.05% aqueous TFA and buffer B consisted of 0.05% TFA in acetonitrile.

All other peptides (Chapters 3, 6 to 10) were purified on a HPLC system comprised of a Spectra Physics SP8700 solvent delivery system and Kratos SF7697 detector and an analytical reversed-phase column (4.6 mm I.D. x 220 mm, Aquapore RP-300, 300 Å pore size and 7.5 µm particle size, Pierce Chemicals, CA.). A linear AB gradient (0.1% B per min) at 1 ml/min, where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile, was used. The sample loads varied between 20 and 50 mg per run (Mant *et al.*, 1987a, Parker *et al.*, 1987). One minute fractions were collected. In order to identify the fractions containing the desired peptide, analytical runs of the various fractions were carried out using the same reversed-phase column with a linear AB gradient rate of 1% B/min. The fractions containing the pure peptide were pooled and lyophilized. Amino acid analysis on a Durran D-500 amino acid analyzer were performed to ensure correct sequence.

PROTEIN PURIFICATION

Myosin subfragment 1 (S1) was prepared by the method of Weeds and Taylor (1975), as modified by Talbot and Hodges (1981). The critical steps in S1 preparation are the chymotryptic digestion and subsequent purification. Therefore, these steps are outlined below. After extraction and washing of myosin from the skeletal muscle mince, it was

precipitated by dialysis against a buffer consisting of 0.12 M NaCl, 0.013 M Na_2PO_4, 0.007 M NaH_2PO_4, 1 mM EDTA, pH 7.0. This was brought to room temperature with gentle stirring and the myosin concentration diluted until it was 15 mg/ml. Chymotrypsin, dissolved in less than 10 ml of the dialysis buffer, was added to the myosin solution to a concentration of 0.05 mg/ml (1:200, w/w). The digestion was allowed to continue for 14 min and was terminated with 0.1M PMSF in 95% ethanol to a final concentration of 0.5 mM. The mixture was stirred for 15 minutes to allow complete inhibition to occur. The solution of S1 and undigested myosin was dialyzed against 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3, pH 7.0. The precipitate was removed by centrifugation at 45,000 x g for 30 min. The supernatant was applied to a DEAE-52 cellulose column (2.8 x 45 cm) which had been equilibrated with the same buffer used in the last dialysis. The S1 was eluted with a 0.2 mM KCl / ml linear gradient. The column was run at 40 ml/hr and fractions were collected every 15 min. Protein peaks were determined by reading the various fractions at 280 nm. The fractions containing S1(A1,A2) were pooled and concentrated on an Amicon ultrafiltration device equipped with a PM-10 filter or by a 40-55% $(NH_4)_2SO_4$ precipitation. The S1 was dialyzed against 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3 buffer, pH 7.0. S1 could be stored in the presence of NaN_3 with no loss of activity for three weeks. For longer storage up to a year, a solution of S1 and sucrose in a 1:5 ratio (w/w) can be frozen rapidly in liquid nitrogen (N_2) and stored at -20° or -70°C. Prior to use, the S1-sucrose solution was extensively dialyzed against ATPase buffer containing 1 mM DTT. The K^+-EDTA ATPase activity for the various S1 (A_1,A_2) preparations varied between 450 and 600 nmole of PO_4^{-2} released/min/mg of S1.

HMM was prepared using the procedure by Weeds and Pope, 1977. The extracted myosin was digested using trypsin (1:400 w/w) for 12 min at room temperature. The digestion was terminated with a trypsin inhibitor. A 0 to 40% $(NH_4)_2SO_4$ precipitation was done to remove light meromyosin (LMM) followed by a 40 to 55% $(NH_4)_2SO_4$

precipitation to remove HMM. The HMM precipitate was dissolved and dialyzed against 1 mM DTT, pH 7.0-8.0. HMM could be freeze-dried as a 10% sucrose mixture for long term storage.

S1 and HMM are enzyme fragments that are difficult to prepare and purify. There are several precautions one should take when dealing with either myosin fragment. The pH of the solutions used throughout myosin extraction should be checked at each step, especially after addition of ATP. At all times the protein solutions should be kept at -4°C. Due to the great sensitivity to metal ions, water should be deionized and distilled. Any contact with metal surfaces should be avoided and when the muscle is initially blended, the time the myosin is in contact with the blades of the blender should be minimal. Finally, dialysis bags should be treated by boiling in a solution of 1 mM EDTA and 20 mM sodium bicarbonate.

Rabbit skeletal Tn, TnI and TnC were prepared by the procedures of Chong and Hodges (1982). Bovine brain calmodulin was prepared as described by Cachia *et al.* (1986). Bovine cardiac TnC was prepared as described by Strapans *et al.* (1972). To prepare Ca^{2+}-free TnC in Chapter 6, TnC was dialyzed against excess EDTA, DTT and chelex. The final dialysis was against the experimental buffer and DTT. To prepare Ca^{2+}-free TnC in Chapter 7, TnC was denatured in 6 M guanidine hydrochloride in the presence of excess DTT and EDTA, then dialyzed against the appropriate buffer. 1 mM DTT was added to the final dialysis. Rabbit cardiac α-TM was extracted and purified as described by Pato *et al.* (1981). G-actin was purified from rabbit skeletal muscle acetone powder as described previously (Spudich and Watts, 1971). The purity of all proteins were checked by sodium dodecyl sulfate urea-polyacrylamide gel electrophoresis (12% crosslinked). The concentrations of all proteins and synthetic peptides were determined by amino acid analyses, except S1 and HMM which were determined by absorbance (Yagi *et al.*, 1967; Young *et al.*, 1964).

SODIUM DODECYL SULFATE UREA-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS gel electrophoresis was done with a 12% acrylamide 6M urea gel using the similar procedure to Weber and Osborne (1969). The gels were run on Protean dual slab cell apparatus (Biorad, Richmond, CA) giving a gel size of 14 x 16 cm. Samples were dissolved in 8 M urea, 0.1 M H_3PO_4-Tris, 1% SDS and 1% βME, pH 6.8. and heated to 60°C for 20 min. A solution of bromophenol blue dye was used for tracking. After electrophoresis, gel were stained with a 0.25% solution of Coomasie brilliant blue dye in methanol, acetic acid and water (1:1:8, v/v/v).

ATPase ASSAYS

ATPase assays were performed using an automatic pH-stat apparatus consisting of a Radiometer TT-2, SBR2C Titrigraph, and SBU 1 ml syringe burette (Côté and Smillie, 1981) for assays done in Chapter 5 or a Brinkman Metrohm 614 Impulsomat, 655 Dosimat, 625 Dosigraph and 635 pH meter with a 1 ml syringe (Van Eyk and Hodges, 1991) for assays done in Chapters 8 and 9. Assay samples, 2 ml in volume, were placed in glass vials and stirred continuously at 25°C. The S1 ATPase activities were measured in a Mg^{2+}-ATPase assay buffer consisting of 5 mM Tris, 30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, 2.5 mM ATP pH 7.8. The HMM ATPase assays were measured in a buffer consisting of 2 mM Tris, 30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, 2.5 mM ATP, pH 8.0.

The purified proteins used in the assays were prepared as follows: tropomyosin and TnC (~3 mg/ml) were dissolved in the Mg^{2+}-ATPase assay buffer (minus ATP, plus 2 mM β-mercaptoethanol). Proteins were dialyzed against this buffer at 4°C. Native Tn (~3 mg/ml) was dissolved in the Mg^{2+}-ATPase assay buffer (minus EGTA and ATP, plus 2 mM β-mercaptoethanol) and dialyzed against this buffer at 4°C. Due to solubility

problems, TnI was dialyzed in 8 M urea, 1 M KCl, 2 mM DTT, 10 mM Tris buffer, pH 7.8. The TnI sample was next dialyzed against 1 M KCl, 2 mM DTT 10 mM Tris buffer, pH 7.8, and then finally against 0.5 M KCl, 2 mM DTT, 10 mM Tris buffer, pH 7.8. The dialysis of TnI was carried out under a nitrogen atmosphere to prevent oxidation of cysteine residues (Horwitz *et al.*, 1979). S1 or HMM was dialyzed against the ATPase buffer. G-actin was polymerized by the addition of 1 M KCl to a final concentration of 30 mM.

The concentrations of the stock protein and peptide solutions were such that dilution of the actin-S1 concentration did not exceed 6% and was usually less than 3%. Thus, the effects of the addition of inhibitory proteins and peptides could not be ascribed to the effect of simple dilution of the acto-S1 concentration. S1 by itself had a low ATPase rate and in acto-TM-S1 ATPase assays never exceeded more than 10% of the initial acto-S1 ATPase rate. Correction was made for the initial S1 activity unless otherwise stated. ATP hydrolysis in the acto-S1 system is linear over a wide range of ATP concentration (Bremel *et al.*, 1972); but when ATP levels fall below 10^{-5} M, S1-ATP rigor complexes can become more abundant and potentiate ATP hydrolysis (this phenomenon occurs only with regulated filaments). Rigor complex formation was avoided, since 2.5 mM ATP added initially to the assay was never exhausted during the course of the assay. The TM to actin ratio used in these experiments was 1:7. The actin and S1 ratios varied with each experiment. To ensure the ATPase assay was functioning correctly, Tn or TnI inhibition and Ca^{2+} dependent release of inhibition was performed with each new S1 preparation.

The continuous titration method was used throughout this thesis (Van Eyk and Hodges, 1986). Due to variations in ATPase assays from day to day, data for each set of experiments are obtained on the same day. Replicates of each set of experiments were performed on different days. This ensured that the results were quantitatively similar.

Continuous Titration Assay

The analysis of the effect of one variable on the acto-S1 ATPase activity was performed employing the continuous titration method. In this procedure, a single assay vial of acto-S1 is titrated with the variable peptide. The effect of accumulated protein or peptide on the ATPase activity was determined after each consecutive addition of the variable protein or peptide [for example, when studying the inhibition of the acto-S1 ATPase by increasing amounts of TnI peptide (Chapter 5)]. This same procedure was used to investigate the effect of two variables on the ATPase activity; for example, ±F-actin and increasing concentrations of actin peptide (Chapter 8). Two sets of assay vials of identical concentrations of S1 but with one set of vials containing F-actin were prepared. Each vial was assayed with a single aliquot of peptide to obtain six separate ATPase activity measurements.

The results, reported as relative ATPase, are in terms of percent of inhibition or activation of the initial ATPase rate. For the individual assay, the percent change in ATPase rate produced by subsequent addition of proteins or cation was calculated directly from change in the ATPase rates. Otherwise, the ATPase rates were reported as s^{-1} which is equivalent to nmole of PO_4^{-2} released/sec/nmole of S1 or HMM.

AFFINITY CHROMATOGRAPHY OF PEPTIDES

Conventional Affinity Chromatography

The synthetic peptides, mastoparan and TnI peptide were coupled to cyanogen bromide activated Sepharose 4B according to the general procedure described by Pharmacia (Axén *et al.*, 1967, used in Chapters 3 and 4). Fluphenazine was coupled in 0.1 M $NaHCO_3$ - 20% (v/v) dioxane (pH 11) to epoxy-activated Sepharose 6B. Reaction was done for 48 h in the dark at 60°C (used in Chapter 4). The column support (epoxy-activated sepharose 6B-peptide)was washed with coupling buffer, distilled water, and

followed with 1 M ethanolamine-H_2O (pH 8.0). To ensure all the residual epoxy sites were blocked, the support was left in 1 M ethanolamine for 4 h at room temperature. The support was washed in water, followed by a buffer of 20 mM Tris, 0.5 M NaCl (pH 7.5).

The three affinity columns ; mastoparan, TnI peptide and fluphenazine (1 mL plastic syringes, 40 x 5 mm I.D.) were run at 4°C, with a flow rate of 10 ml/hr. The buffer (10 mM Mops, 100 mM NaCl, 0.1 mM EDTA, pH 7.2) was mixed batchwise with Chelex 100 (Biorad) for 2 h to remove any divalent metal ions present. EDTA (0.1 or 0.01 M), $CaCl_2$ (2 mM), or $MgCl_2$ (5 mM) was added to obtain the buffers with the absence or presence of various metal ions. The buffers were stored in plastic containers which had been pre-rinsed with an EDTA solution. Fractions (1.6 mL) were collected and the absorbance was read at 278 nm. The identification of the eluted protein peak was confirmed by running sample from the peaks on 12% SDS - 6M urea-polyacrylamide gel electrophoresis. Controls were run in which a third buffer containing 6 M urea, 10 mM Mops, 0.1 mM EDTA, pH 6.2 was run following the EDTA buffer to ensure all the CaM or TnC had been previously eluted from the affinity column.

High Performance Affinity Chromatography (HPAC)

The skeletal or cardiac TnC or skeletal TnI affinity column was prepared using the standard procedure for the 50 x 4.6 mm I.D. Beckman Ultraffinity-EP column. To maximize attachment of TnC or TnI by amino groups and to minimize sulfhydryl attachment to the column support, 1 M potassium phosphate derivatizing buffer, pH 7.5, was used (for more details see Chapter 3). In addition, 2 mM $CaCl_2$ was included when TnC was derivatived. A 10 to 20 μM solution of protein (10 ml) was recycled for 48 h at 0.2 ml/min to achieve maximum derivatization. Undervatized epoxy groups were blocked with βME. The columns were stored in 0.5 M KCl, 0.1% NaN_3 pH 6.0 at 4°C.

In Chapter 3, a variety of flow-rates and gradient rates were used to determine the effect of these parameters on the elution profile. In Chapters 5 and 10 where retention

times of various peptide analogs are compared, peptides (10 nmole) were eluted by applying a linear AB gradient of 1% B/min (equivalent to 10 mM KCl/min), 1.5% B/min (equivalent to 15 mM KCl/min) or 2.0% B/min (equivalent to 20 mM KCl/min), where buffer A was 20 mM Tris, 0.1 mM EGTA, pH 6.9, containing either 3 mM $MgCl_2$ or 1 mM $CaCl_2$ and buffer B was buffer A plus 1.0 M or 4 M KCl. For elution of actin peptides four additional step gradients were used once 200 mM KCl was reached using the 15 mM KCl/min gradient . These step gradients consisted of the starting buffer plus either 1 M, 2 M, and 4 M KCl and finally 6 M urea. The flow rate was 0.5 ml/min and the effluent monitored at 210 nm with 0.2 absorbance units full scale. The molarity of KCl required for elution of the peptides was determined by subtracting the gradient delay time (tg) from the elution time of the peptide followed by multiplication of the gradient rate (mM salt/min). The tg is defined as the time for the gradient to reach the detector from the proportioning valve via pump, injector loop, and column (Guo *et al.*, 1986). The tg was taken to be equivalent to the time elapsed from the start of a linear AB gradient to an observed off scale absorbance due to the β-mercaptoethanol (3 mM) included in buffer B. A tg of 5.5 to 5.8 min was determined for the HPLC systems employed in the TnC and TnI affinity studies, respectively.

SIZE EXCLUSION CHROMATOGRAPHY

Mixtures of protein and peptide were incubated at room temperature for 10 minutes (Chapter 8 and 10). The peptide-protein mixtures were run on a high performance size-exclusion column, Altex TSK G2000 SW (7.5 mm I.D X 30 cm) (Beckman Inc., Berkeley, CA) in a buffer consisting of 10 mM Tris, 100 mM KCl, in the presence or absence of 2 mM $CaCl_2$, pH 6.8 at a flow rate of 0.5 ml/min. The salt was present in the buffer to eliminate non-specific ionic interactions between the peptide or protein and the support, and hence maintain ideal size-exclusion behavior (Mant *et al.*, 1987b). One minute fractions or individual peaks were collected and lyophilized for further analysis.

The HPLC system used for the size-exclusion chromatography (SEC) was a Vista series 5000 liquid chromatograph (Varian, Walnut Creek, CA), HP 1040A detector, 8513 computer, HP9121 disc drive, HP25A inkjet printer and HP7470A plotter (Hewlett Packard, Palo Alto, CA).

Analysis of the quantity of peptide present in the various size-exclusion fraction was determined from radioactivity measurements on a Beckman Scintillation counter. Analysis of the proteins were done by reversed-phase chromatography on a hydrophobic-interaction column (HIC) for S1 (Chapter 8) or a microbore RP column for Tn (Chapter 10). The HIC column used to separate and quantitate the S1 was a Bio-Gel Spherogel TSK-phenyl-5-PW column (7.5 mm I.D. X 7.5 mm, Biorad). This column was operated in reversed-phase mode using a linear AB gradient (2% B/min) where solvent A consisted of 0.05% aq. TFA and solvent B was composed of 0.05% TFA in acetonitrile at a flow rate of 1.0 ml/min (Ingraham *et al.*, 1985). Under these conditions, the S1 heavy chain was resolved from the light chains A1 and A2 which are associated with the native S1 molecule. SDS urea gel-electrophoresis was run on the fractions collected from this column to confirm the location of S1 heavy chain and the light chains. Radioactivity measurements were done in order to detect the presence of peptide in each fraction collected from the HIC column. The area of the S1 peak was quantitated to determine the relative amount of S1 present in each SEC fraction. The reversed-phase chromatography on the HIC column was carried out on an HP1090 fully automated liquid chromatograph containing a HP8513 computer, HP9133 disc drive, 9000-300 hard disc and HP 7470A plotter. The microbore reversed-phase column used to separate the Tn subunits (TnT, TnC and TnI) from the peptide in each peak collected from the SEC run was a Brownlee Aquapore RP-300 column (1.0 mm I.D. x 100 mm, 7 u particle size and 300 A pore size) A linear AB gradient (2% B/min) was used where solvent A consisted of 0.05% aq. TFA and solvent B was composed of 0.05% TFA in acetonitrile at a flow rate of 0.1 ml/min.

CENTRIFUGATION STUDIES

Mixtures of S1 (4 or 6.8 nm) with increasing quantities of peptide were incubated at room temperature for 10 min and then centrifuged for 15 min at 15,000 rpm at 4°C (Chapter 8). There was no difference in the amount of S1-actin 1-28 peptide precipitated if 0, 10 or 30 minute incubation were done. The assay was done in the Mg^{2+}-ATPase buffer described for the S1 and acto-S1 ATPase assays except ATP was not present. Aliquots of the supernatant were taken for radioactive measurements to quantify the actin peptide remaining in the supernatant and for amino acid analysis to determine quantity of S1 in the supernatant. From these values the quantities of S1 and peptide in the pellet were determined.

To determine whether actin peptide could bind to acto-S1 complex, centrifugation studies were performed as described above except actin (5.4 nm) was present in the S1 (5.1 nm) solution (assay volumn of 1.5 ml, Chapter 8). In the absence of peptide and S1, 87 to 90% of the actin was pelleted. In the absence of peptide, approximately 100% of the S1 was found in the actin pellet. Centrifugation was carried out for 25 min at 15,000 rpm at 4°C. The quantity of radiolabelled actin peptide found in the supernatant following centrifugation was determined by radioactive measurements. From these values the amount of peptide present in the pellet (comprised of actin or actin-S1) was determined. Amino acid analysis and SDS urea gel electrophoreses of the supernatant was done to ensure the quantity of actin and S1 remained constant as increasing quantities of actin peptide was added.

SKINNED MUSCLE FIBRE ASSAYS

Skinned rabbit psoas fibres were prepared as described by Kawai and Schulman (1985) and cardiac fibre bundles (about 4 mm in length and 0.1 mm in diameter) prepared from porcine trabecula septomarginalis were extracted for two days in a solution containing

50% glycerol and 50% of a buffer containing 20 mM imidazole 10 mM NaN_3, 5 mM ATP, 5 mM $MgCl_2$, 4 mM EGTA, 2 mM DTE, 1% Triton-X-100, pH 7.0. Subsequently, the cardiac and psoas preparations were stored in the same solution, but without Triton-X-100 at 4°C for several days, as described by Herzig, Feile and Rüegg (1981). After mounting the preparations with a fast-setting glue on an isometric force transducer (type AME 801, SensoNor, Norway) and a glass rod attached to a micro manipulator, the fibres were relaxed by immersion into a solution containing 30 mM imidazole, 10 mM ATP, 10 mM creatine phosphate, 5 mM NaN_3, 5 mM EGTA, 12.5 mM $MgCl_2$ and creatine kinase 380 U/ml, pH 6.7. Ionic strength was adjusted to 80 mM with KCl, t=20°C. Contraction was induced by immersion into an analogous solution in which EGTA was replaced by a CaEGTA buffer. In studies on skinned psoas fibres, 6 mM inorganic phosphate were also added. The buffered calcium ion concentration was determined from the ratio of EGTA to CaEGTA, essentially according to Portzehl *et al.* (1964) but using an apparent dissociation constant of 1.6 µM for the CaEGTA buffer at pH 6.7, 20°C (Blinks *et al.*, 1982).

CIRCULAR DICHROISM SPECTROSCOPY

The CD experiments were conducted at 25°C on a JASCO J-500c spectropolarimeter fitted with a thermostated cell holder and interfaced with a JASCO DP-500N data processor (Chapter 6 and 7). The pCa curve of TnC (or IC) in the absence of peptide was directly compared to the pCa curves obtained in the presence of synthetic peptide using the same protein sample,buffer and.Ca^{2+} solution. In Chapter 7, the pCa curve for skeletal and cardiac TnC were repeated numerous time and had an experimental error of less than 0.13 and 0.09 for the low and high affinity Ca^{2+} binding sites, respectively. Experiments were carried out in 1000 µl volumes with Ca^{2+} added in aliquots to a total volume of 1030 µl. The concentrations of the cardiac and skeletal TnC varied between 22-30 µM. One molar equivalent of peptide was present.

Data was expressed as observed ellipticity θ (millidegrees), and the error in the observed value was ± 0.2 mdeg. The ellipticity values at 222 nm were converted to mean residue ellipticity values by using the following equation:

$$[\theta]_{222}=\theta M_r/100LC$$

where θ = observed ellipticity (degrees), L = cell path length (decimeters), C = concentration in mg/ml and M_r = mean residue molecular weight calculated from the appropriate amino acid sequences. The mean residue ellipticity values have an error of approximately ± 300 mdeg. The theoretical curves were calculated by the summation of $[\theta]^{222}_{TnC} + [\theta]^{222}_{peptide}$ at each Ca^{2+} concentration. The fraction helix , f_H, was estimated using the empirical equation obtained by Chang et al. (1978):

$$f_H=[\theta]_{222}/[\theta]^{\infty}_{H}(1-k/n)$$

where $[\theta]^{\infty}_{H}$ = mean molar residue ellipticity for an infinite helix (-37,400°), k = chain length dependence factor (2.5) and n = the number of residues in a typical helix (9). The number of helical residues was then calculated by multiplying f_H by the total number of amino acid residues in the protein or protein-peptide complex. The error in the number of helical residues in TnC (in the absence or presence of peptide) was calculated and ranged between 0.7 to 1.8 helical residues with an average of 1.33 residues. Therefore, any change in the number of helical residues induced by the peptide binding to TnC greater than 2 residues is significant

FLUORESCENCE SPECTROSCOPY

The intrinsic Tyr fluorescence was determined using a Perkin Elmer MPF-44B fluorescence spectrophotometer, equipped with the DCSU-2 corrected spectra accessory which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode and thermostatted cells were maintained at 20°C. Detection of

fluorescence was effected at 90° to the excitation beam. The emission and excitation wavelengths were 301 and 277 nm, respectively (Lakowicz, 1983). In Chapter 7, the pCa curves obtained in the absence of peptide was directly compared to the pCa curve of TnC using the same protein sample, buffer and Ca^{2+} solution. The pCa curves for skeletal and cardiac TnC were repeated numerous times and had an error of less than 0.13 and 0.09 for the low and high Ca^{2+} affinity sites. The initial volume of sample was 1000 µl and Ca^{2+} aliquots were added to the final volume of 1030 µl. The concentrations of cardiac and skeletal TnC varied between 11 and 15 µM, to which one mole equivalent of peptide was added.

DETERMINATION OF Ca^{2+} BINDING CONSTANTS AND HILL COEFFICIENTS

The experimental data obtained from circular dichroism and fluorescence spectroscopy studies were analyzed using a computer software program designed to determine biphasic binding curves (program provided by Dr. B.D. Sykes, University of Alberta, Chapter 7). The fitting program analyzed data in the form of 2 Hill coefficients:

$$Z = \frac{[Ca^{2+}]^{n1}}{[Ca^{2+}]^{n1} + Kd_1{}^{n1}} + \frac{[Ca^{2+}]^{n2}}{[Ca^{2+}]^{n2} + Kd_2{}^{n2}}$$

where Z is the number of Ca^{2+} bound (occupancy), Kd_1 and n1 are the dissociation constant and Hill coefficient for the low affinity binding sites, respectively and Kd_2 and n2 are the dissociation constant and Hill coefficient for the high affinity Ca^{2+} binding sites. The Chi value, which measures the deviation of the experimental values from the values of the best-fit curve, ranged from 0.92 to 2.05, with an average value of 1.22. These values are less than the Chi value (3.84) required for a level of significance of below 0.05, in other words the experimental data is within the 95^{th} percentile of the chi squared distribution.

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CHAPTER 3

HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY (HPAC) OF PEPTIDES AND PROTEINS

INTRODUCTION

High-performance affinity chromatography (HPAC) is used throughout this thesis to investigate the interactions between synthetic peptides of biologically important regions of muscle proteins and their target proteins (Chapters 5 and 10). Since HPAC has only been recently developed, this Chapter deals with the optimization of running conditions when using peptides and/or proteins as solute or ligand molecules. This chapter is published in a book entitled "High Performance Liquid Chromatography: Separation, Analysis and Conformation", 1991 (Mant, C.T. and Hodges, R.S., eds), CRC Press Inc., pgs 479-491.

All methods of chromatography exploit physical or chemical differences between molecules in order to achieve their separation. In the case of affinity chromatography, separation is based on the biological properties of the molecule(s), in particular, their relative binding affinities for a specific ligand bound to an inert column support. The choice of ligand is critical, and is the main determinant for achieving the extreme specificity of which this chromatographic method is capable. The advantage of affinity chromatography over other chromatographic methods is this inherent biospecificity, coupled with the option of employing nondenaturing conditions during the separation.

Affinity chromatography is a powerful technique for both investigating the molecular properties of protein-protein or peptide-protein interactions and for purifying proteins. Since the advent of high-performance affinity chromatography (HPAC) (Ohlson *et al.*, 1978), which dramatically decreases the time required for each chromatographic

separation compared to conventional affinity chromatography, the routine identification and quantitation of minute amounts of protein(s) from complex mixtures of proteins and peptides has become possible.

GENERAL PRINCIPLES OF HPAC

The overall processes involved in affinity chromatography are based on the bioselective interaction between the molecule(s) of interest in the sample mixture and the ligand which is attached to an inert column support. Figure 6 is a schematic representation of the basic procedures involved in achieving separation of molecules by affinity chromagraphy. A sample which contains both the molecule of interest and impurities is loaded onto the affinity column (Step 1); adsorption of the solute molecule occurs upon binding to the ligand, while the impurities are washed through the column (Step 2); the solute molecules are desorbed from the ligand by a competing desorption agent present in the dissociating buffer (Step 3); finally, the column is then regenerated (Step 4) for the next separation. At each step in the separation, there are numerous conditions which must be optimized for each particular separation; thus, the choice of ligand and its chemical attachment to the inert column support will dictate the type of column that can be used. The type of desorption agent and whether it is applied as a step gradient or as a linear gradient will determine the efficiency of the separation. The choice of flow-rate will affect peak width, peak height and peak elution volume.

ADVANTAGES OF HPAC OVER CONVENTIONAL AFFINITY CHROMATOGRAPHY

The main advantage of HPAC over conventional affinity chromatography is the increased speed during the loading, elution and regeneration steps of the separation. This is achieved as a result of the small particle size of the silica or polymeric packing used in HPAC compared to that used in conventional soft gels. The small diameter of the particles (~10 μm) results in increased mass transfer rates that allow the solute to move in and out of

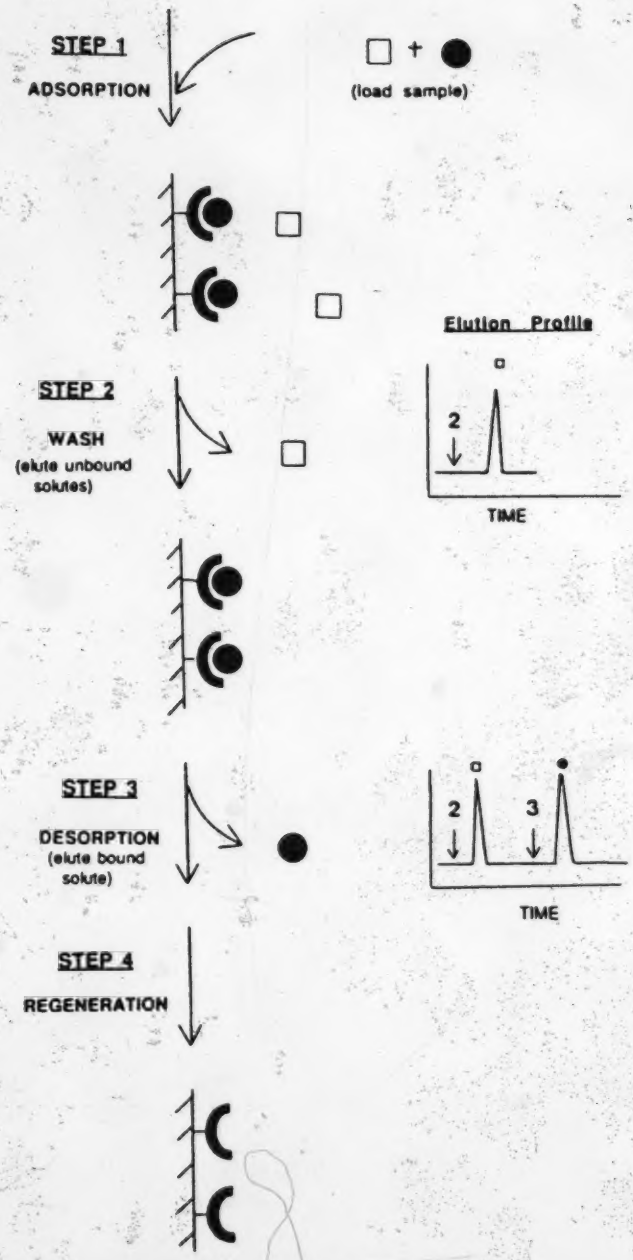

Figure 6. Schematic representation of affinity chromatography. Step 1: Sample is loaded onto the affinity column. The solute molecule is biospecifically adsorbed onto the column when it binds to the ligand-support. Step 2: The sample impurities are washed through the column and detected as the first peak. Step 3: The solute molecule is released from the ligand by the dissociation (or desorbing) buffer containing a desorbing agent. The second peak will contain the pure solute molecule. Step 4: The affinity column is regenerated and is ready for the next separation.

the matrix more rapidly. This means that the dynamic sample capacity of the column (the capacity of the column when flowing) is maintained, even at high flow rates. Hence, the small particle size used in HPAC increases the efficiency of the column, not by increasing the number of theoretical plates as with other chromatographic methods, but rather by increasing column capacity. Flow-rates up to 140 times faster than conventional columns can be used with HPAC. However, since the capacity of the column and efficiency of the ligand-molecule interaction depends on the diffusion rate of the solute molecule(s) plus its association rate with the ligand, the capacity of the column may be reduced at very high flow-rates. Excessively high flow-rates are not normally used because of large column backpressures which may damage the immobilized ligand. Hence, most affinity chromatographic procedures utilize flow-rates of 0.5 to 1.0 ml/min for analytical columns, where sample capacity is less affected by flow-rate. It is especially important during purification or quantitation of proteins that the sample capacity of the column be greater than the quantity of the solute molecule in the sample. Though lower flow-rates can increase sample capacity, it should be ensured that the total quantity of sample is able to bind to the ligand and not go through the column along with the impurities. The sample capacity of a column can also be affected by the quantity of the ligand derivatized on the column.

Due to the reduced time for each separation in HPAC, multiple runs can be carried out in a single day, thus eliminating any day-to-day fluctuations in results. This is a particular concern when comparing quantities isolated from different samples or investigating the elution times between various related solute molecules. The reproducibility between runs is high in HPAC. For example, three consecutive runs of peptide binding to a protein affinity column showed a reproducibility within 0.1 minutes in peptide elution over a 30-min run time (Van Eyk and Hodges, 1988).

CHOICE OF LIGAND AND LIGAND ATTACHMENT

There are two classes of ligands that can be used in affinity chromatography: ligands can be specific for a particular molecule or may be multifunctional and bind a number of related molecules. Employing the second class of ligand depends on both the sample and experimental application. A ligand must possess the following properties: bind reversibly to the solute molecule of interest, be stable under conditions required to dissociate the molecule, and possess high specificity for the solute, i.e., not bind impurities that might be present in the sample. Equally important, the ligand must contain a functional group that can be used to couple it to the column support such that it does not interfere with the solute binding site. Thus, when the ligand is bound to the support, its binding site for the solute is unaffected.

The chemistry of ligand immobilization to the stationary support is critical, since the interaction with the solute molecule may be affected by conformational changes or steric hindrance induced in the ligand upon attachment to the support. Unfortunately, at the present time there is not a large choice of matrix functional groups on commercially available underivatized HPAC supports. Most researchers are restricted to using the commercially prepacked HPAC columns that are available as either activated supports (ready for coupling the ligand of choice) or as ready to use matrixes that have commonly used ligands (e.g., Protein A) already coupled to the matrix. Table 3 lists several manufacturers and underivatized columns which are available.

There are a number of different matrix functional groups available which will bind amino, hydroxyl or sulfhydryl groups of amino acids on the ligand. The most versatile is the epoxy functional group. Figure 7 illustrates the chemistry of epoxide linkages of a ligand to a silica support. Although epoxy groups bind preferentially to sulfhydryl groups, there is some selectivity towards various other functional groups (e.g., hydroxyl and

amino) on the ligand, depending on the pH of the derivatizing buffer. Thus, when derivatizing a column with a protein ligand, a certain percentage of the protein ligands will be attached to the support by different reactive groups. In addition, since hydroxyl (OH) and amino (NH_2) groups are prevalent in an amino acid sequence, a protein would be attached at different sites along its sequence. This is not usually a problem, even if some ligand-support linkages occur at the ligand-solute binding site, since the majority of attachment sites will be at sites other than that of the ligand-solute interaction. Under acidic conditions, the protonated epoxide group (the mechanism involves an active protonated oxygen) is extremely reactive and will react with nucleophiles. The relative reactivity is SH > OH > NH_3^+. To promote amino linkages, the pH is increased and/or non-aquous solvent (eg., acetronitrile) is used so that the epoxy group is not protonated; also, NH_2 is a stronger nucleophile than NH_3^+. It should be noted that, since supports that couple only to primary amines will bind at numerous sites located throughout the protein, the number of ligands on the column able to interact with the molecule of interest may be lowered.

Ligands of small molecular weights, such as peptides, pose additional problems during ligand-support attachment. Locating the functional group involved in the binding of the ligand to the support away from the actual binding site for the solute molecule is difficult due to the small size of the peptide. Ideally, it should be at either the amino or carboxyl terminus of the peptide, whichever is located furthest away from the amino acid residues involved in solute binding. Thus, if synthesizing a peptide to be used as an affinity chromatographic ligand, a cysteine linker could be added at either terminus of the sequence, if there is no cysteine already in the sequence, for reaction with the epoxy functional group on the support. Even so, the closeness of the ligand attachment site to the solute binding site can lead to the disruption or inaccessibility of the solute for the ligand. The use of spacer arms in order to expose the small peptide to the solvent is commonly used in conventional affinity chromatography. Although the number of commercially

Table 3. Examples of Underivatized HPAC Columns

Manufacturer	Column name	Ligand specificity	pH range	Pore size (Å)	Particle size (μm)	Support material	Support functional group
Beckman	Ultraffinity-EP	SH NH_2 OH	2-7	-	10	silica	epoxy
Biorad	Affi-Prep 10	NH_2	2-10	N/A	N/A	polymer	N-hydroxysuccinimide
Pierce	SelectiSpher-10	NH_2	<8.5	300	10	silica	tresyl
Separation Industries	NuGel H-AF Polyepoxy	SH NH_2	2.5-8.5	300	10	silica	epoxy
Separation Industries	NuGel H-AF Polybond	NH_2	2.5-8.5	300	10	silica	hydroxyl

available HPAC underivatized columns are limited, Nu-Gel H-AF polyepoxy support, manufactured by Separation Industries, has a long (12Å) spacer arm attached to a silica support. When dealing with a peptide/protein interaction, it is recommended to use the protein as the ligand and the peptide as the solute molecule. In so doing, attachment problems are minimized. However, if it is essential for a peptide to be a ligand, the above concerns should be kept in mind. An example where a peptide was successfully used as a ligand is with a synthetic peptide of a region of troponin I (TnI) which was used to bind troponin C (TnC; MW = 17,960 daltons) (Van Eyk and Hodges, 1987). The sequence of the TnI peptide is Ac-G^{104}-K-F-K-R-P-P-L-R-R-V-R^{115}-amide. The conventional CNBr Sepharose matrix, which binds to primary amines, was used, but a HPAC column could have been employed. The coupling of the peptide to the matrix was through the ε-NH_2 of the Lys residues at positions 105 and 107. Since the peptide when bound to the support still bound TnC, the residues of the peptide important for binding this protein were situated distal to the residues involved in ligand-support attachment (Van Eyk and Hodges, 1988). Ni and Klee (1985) also reported an interesting example of using peptides ligands, when they coupled calmodulin tryptic fragments to Sepharose 4B. This affinity packing was then used to test the ability of different calmodulin-regulated enzymes to recognize different domains of calmodulin.

Another concern when derivatizing a ligand is that its concentration on the support can be reduced if either the ligand or the solute molecule has a large molecular weight, since the larger physical size of the proteins increases the chance of steric hindrance between the ligands bound on the support, or the solute molecules once bound, if the ligands are situated close to each other. The neighboring ligands will interfere with the solute binding and, hence, lower the efficiency of the column. Reduction of ligand concentration may also be necessary if the ligand-solute dissociation constant is very low ($k_d < 10^{-9}$), necessitating a higher concentration of desorbing agent to elute the solute molecule. This is

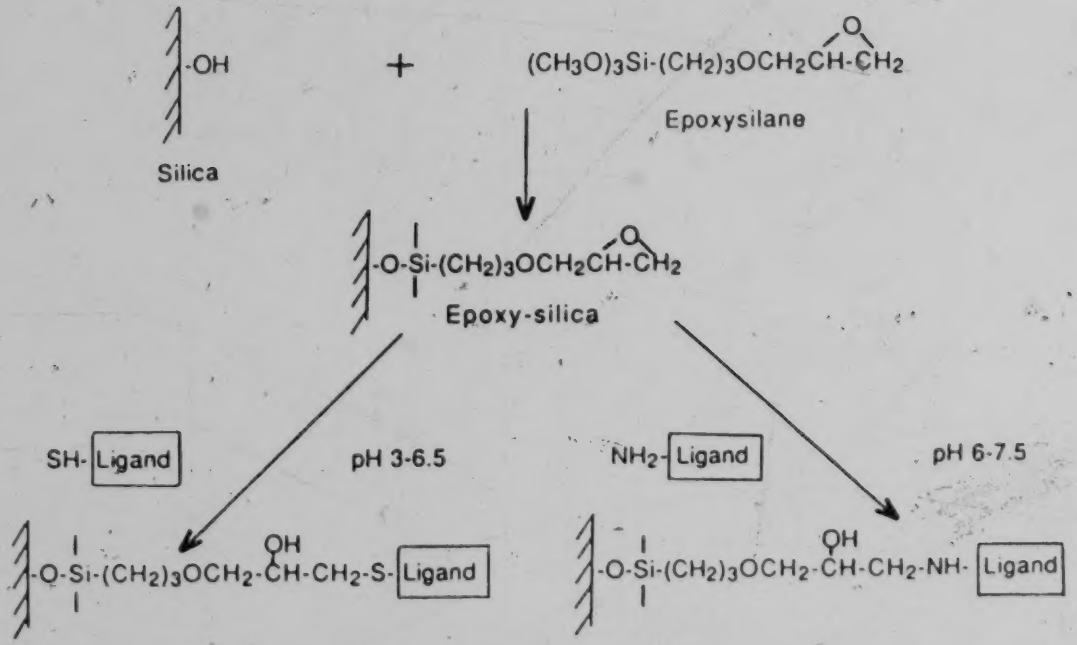

Figure 7. The chemistry of ligand derivatization to epoxy-silica matrix. Silica is reacted with epoxysilane to produce epoxy-silica, which is available commercially. Epoxides are highly reactive due to the ease of opening the highly strained 3-membered ring. A nucleophilic group on the ligand attaches the epoxide. At low pH, the epoxide is protonated and is very reactive. In contrast, at more alkaline pH, the nonprotonated epoxide requires a stronger nucleophilic agent (i.e., NH_2) for the reaction to occur. Hence, some degree of selectivity of ligand functional group attachment to the support can be achieved.

a consideration only if the desorption agent is a peptide or protein and a lower concentration of this agent is desired (especially if the counter ligand is expensive or in short supply). In addition, if the interaction between the solute and ligand is strong, the dissociation of the solute from the column will occur slowly. This leads to broader peaks regardless of whether step gradient or linear gradient elution is employed. Hence, the volume of eluting buffer required for elution of the solute would increase. By reducing the ligand concentration, elution volumes will be minimized, a particular advantage during analytical applications. Unreacted functional groups on the support (not attached to ligand) may be blocked by β-mercaptoethanol or glycerol, which will increase the column hydrophilicity but not alter the net charge of the ligand-support. Blocking functional groups with ethanolamine, which adds a cationic charge, and glycine, which is an ampholyte, will contribute unwanted ionic interactions. It should be noted that reducing the ligand concentration on the support will also reduce the maximum sample capacity of the column. Thus, for preparative purifications, larger amounts of derivatized support will be required compared to analytical applications. Finally, if a solute-ligand complex has a high dissociation constant ($>10^{-7}$), it is desirable to maximize ligand concentration on the support.

RUN CONDITIONS IN HPAC

A. Choice of buffers

All the equilibrating, running or adsorption buffers must be nondenaturing. This will ensure that the ligand and/or solute molecule(s) retain their biospecificity.

B. Sample recovery techniques

1. Choice of desorption agent and elution conditions

Optimization of the desorption step in the affinity separation offers the greatest flexibility in customizing HPAC for the system being studied. The elution of the protein or

peptide bound to the ligand involves breaking the same types of bonds that are involved in maintaining the native conformation of proteins. Thus, the conditions chosen to disrupt ligand-solute interactions must be mild to minimize the denaturation of the ligand and solute molecules. Depending on the ligand and solute molecules, either a general (non-specific) or specific agent can be used to dissociate the solute molecule.

There are numerous nonspecific desorption agents that disrupt the interaction between the ligand and solute molecule, including altering the pH or addition of chaotropic ions. Reducing or increasing the pH of the buffer will eliminate ionic interactions between the ligand and solute molecule. It must be remembered, that low pH may denature proteins and high pH is harmful to the silica matrix. A common example of using low pH in the desorption step is in the purification of antibodies on a protein A derivatized column (many companies can supply a ready-to-use protein A column). The bound IgG is eluted by dropping the pH of the same buffer used in the loading step from pH values above neutrality to pH values in the range of 2-3 (Ohlson and Wieslander, 1987). It has been reported that low pH reduces the life of the column (Josic *et al.*, 1987). Also, sudden drops in pH can be harmful to column life and it has been suggested that a pH gradient is less harmful. There are many books and review articles that deal with the complex area of purification of IgG molecules from sera, ascites fluid or cell culture media (Nau, 1989). Chaotropic agents are used to destabilize ligand-solute interactions by disrupting the structure of water and hence, reducing hydrophobic interactions between the ligand and solute. As well, the high salt concentrations will reduce ionic interactions and promote hydrophobic interactions. However, very high salt concentrations are usually required to promote significant hydrophobic effects (for example, 2M ammonium sulfate is required in hydrophobic interaction chromatography). Unlike during pH manipulation, there is no denaturation of ligand or solute, except at very high concentrations of the chaotropic agent, and, thus, this is the desorption method of choice. The effectiveness of chaotropic salts is

as follows: $CCl_3COO^- > SCN^- > CF_3COO^- > ClO_4^- > I^- > Cl^-$. The use of a chaotropic agent was described by Van Eyk and Hodges (1988, also see Chapter 5), in which the relative binding affinities of a series of analogs of the TnI peptide (region 104-115, as described above) were determined on a TnC HPAC column (Figure 8). A linear KCl gradient was applied and the concentration of KCl required to elute the various peptide analogs was a reflection of the binding strength of the peptide-TnC interaction.

Specific desorbing agents that compete with the solute molecule for the same binding site on the ligand can be very useful in purification of a protein that is extremely sensitive to denaturation. Also, the binding specificity of the solute for the ligand can be determined by its competition with other solute molecules for the ligand and monitoring the elution of a solute molecule first attached to the ligand. For example, a 28-residue synthetic peptide from the N-terminal region of actin binds to the protein TnI HPAC column (Chapter 10). The actin peptide can be eluted with a KCl gradient, or by injecting a competing desorbing molecule, such as holo actin (unpublished data). A slight twist to this approach is to have the same molecule as the ligand in the dissociation (desorbing) buffer so that it is able to compete with the ligand for the bound solute molecule (in the example above TnI would be used in the desorbing buffer). In this case, the molecule will be eluted as a complex with the competing ligand. For example, the synthetic actin peptide could be eluted off the TnI HPAC column by injecting TnI or synthetic TnI peptide (104-115) (Chapter 10; Van Eyk et al., 1991). Under certain circumstances, a solute protein may bind sc tightly that denaturation of the ligand is required to elute the bound solute molecule. For example, TnI could not be eluted from the TnC affinity column in 4 M KCl, and it has been reported that the TnI-TnC complex in the presence of calcium is not dissociated by 6 M urea (Chong and Hodges, 1981). A solution of TnC (2 mg/ml) was pumped through the HPAC column at 0.2 ml/min for 1 h. The TnI that was bound to the column was eluted as the TnC-TnI complex.

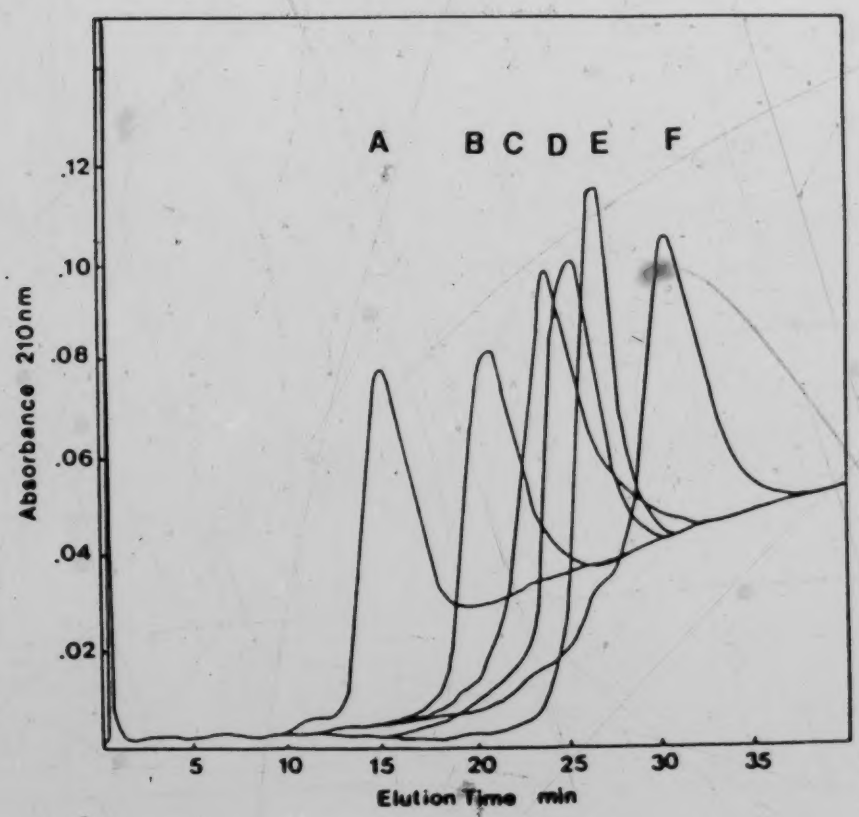

Figure 8. Comparison of retention times of single and multiple glycine-substituted troponin I peptide 104-115 analogs on a troponin C HPAC column. Individual peptide analogs were chromatographed on a Ultraffinity column derivatized with rabbit skeletal troponin C. Linear AB gradient elution was employed to elute the analogs, where buffer A is 20 mM Tris, 0.1 mM EGTA and 1 mM $CaCl_2$, pH 6.9, and buffer B is buffer A plus 1 M KCl. Flow-rate: 0.5 ml/min. Analogs: single substituted, Gly 105 (E), Gly 107 (D), Gly 108 (C), double substituted Gly 105-107 (B) and triple substituted, Gly 105-107-108 (A) and the native troponin I peptide with the sequence Ac-G-K-F-K-R-P-P-L-R-R-V-R-amide (F).

2. Application of non-specific desorbing buffers

Non-specific desorbing agents, such as H^+ ions or chaotropic salts, can be applied either as a step gradient or as a linear gradient. Figure 9 shows the effect of the desorbingbuffer which contains KCl (Buffer B) on the elution profile of the synthetic TnI peptide during step gradient elution from a TnC HPAC column. An initial delay time after loading the sample in the starting (or loading) buffer (Buffer A) was required to wash impurities through the column prior to isocratic application of the desorbing buffer (Buffer B). In Panels A and B, there was an initial 10-min delay, followed by an isocratic step of either 200 mM or 400 mM KCl, respectively. In Panel B, the concentration of KCl in the isocratic step was greater than needed to elute the peptide from the column. Therefore, when the 400 mM KCl front reached the column [at 23.3 min = t_d + 10 min delay; t_d, the gradient delay time, is the time required for the gradient (or KCl front, in this case) to reach the top of the column from the solvent proportioning valve] the peptide was immediately eluted as a sharp peak. In Panel A, the concentration of KCl in the desorbing buffer (200 mM) was low enough to effect a slow dissociation of the solute with the ligand and/or reassociation of the solute with the ligand as the solute molecule travelled down the column. This resulted in peak broadening and a longer retention time after the KCl front had reached the column.

Panels C and D in Figure 9 illustrate the effect of linear gradient elution of the TnI peptide from the TnC HPAC column at gradient-rates of 5 mM and 15 mM KCl/min, respectively, following a 10-min delay. Regardless of the gradient-rate, the peptide was eluted at the same KCl concentration (~180 mM), so that, at the slower gradient-rate, the retention time increased. Also, at the slower gradient-rate, the peak was broadened, since the gradient was not steep enough to eliminate the reassociation of peptide as it travelled down the column.

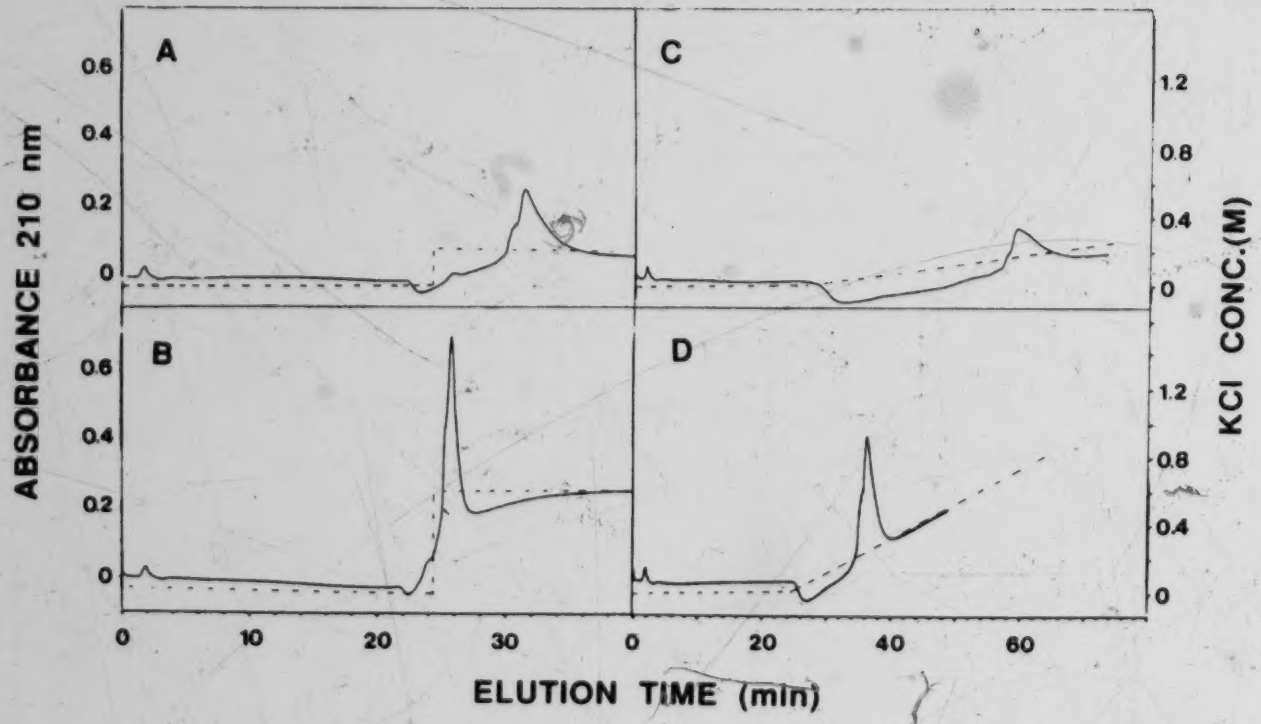

Figure 9. Comparison of step gradient elution and linear gradient elution of troponin I peptide from a troponin C HPAC column. The column, and mobile phases are described in Figure 8. Panels A and B: step gradient elution using 200 mM or 400 mM KCl, respectively, as the desorbing agent. Panels C and D: linear gradient elution at 5 mM or 15 mM KCl/min, respectively. The dotted line shows the concentration of KCl with time under the various elution conditions. Flow-rate: 0.3 ml/min. All runs had an initial 10-min delay time following sample application (10 nmol peptide) and prior to the start of the linear gradient or step gradient KCl front.

Either type of gradient can be easily programmed on an HPLC instrument, by altering the amount of B buffer with respect to A buffer over time, where A is the starting (or loading) buffer and B is the desorbing buffer. Linear gradients are easier from the standpoint that one does not need to have an approximate idea of the conditions required for elution of the molecule, as one does with a step gradient. Therefore, the use of linear gradients is a more universal approach and can be used very effectively for any application.

C. Flow-rates

Flow-rates affect the mass transfer properties of the column and, hence, affect the retention time and peak shape. At reduced flow-rates, the retention time of a peak will be greater due to the increase in the gradient delay time (t_d). The value of t_d can be determined for a particular HPLC instrument by noting the time required to observe an off-scale absorbance, due to the addition of β-mercaptoethanol (3 mM) to buffer B in the absence of a column. The value of t_d at any flow-rate can be calculated as a proportion of the observed t_d at a particular flow-rate. For instance, the HPLC system used to obtain the elution profiles shown in Figure 9 had a t_d value of 4.6 min at 1 ml/min; thus, at flow-rates of 0.5 and 0.3 ml/min, the t_d values will be 9.2 min and 15.3 min, respectively.

Peak shape of the eluted peptide is altered by varying the flow-rate. Figure 10 shows the effect of various flow-rates (0.3, 0.5 and 1 ml/min) on the elution profile of the TnI peptide from a TnC HPAC column. These profiles were obtained by step gradient (Panels A-C) or linear gradient (Panels D-F) elution. Peak characteristics (retention time, peak width, etc.) obtained from these runs are reported in Table 4. A number of observations can be made concerning the results presented in Figure 10 and Table 4. Thus, at constant gradient-rate (20 mM KCl/min) during linear gradient elution, or constant ionic strength (200 mm KCl) of the desorption buffer during step gradient elution, peptide retention time increased, as expected, with decreasing flow-rate in both elution modes; peak

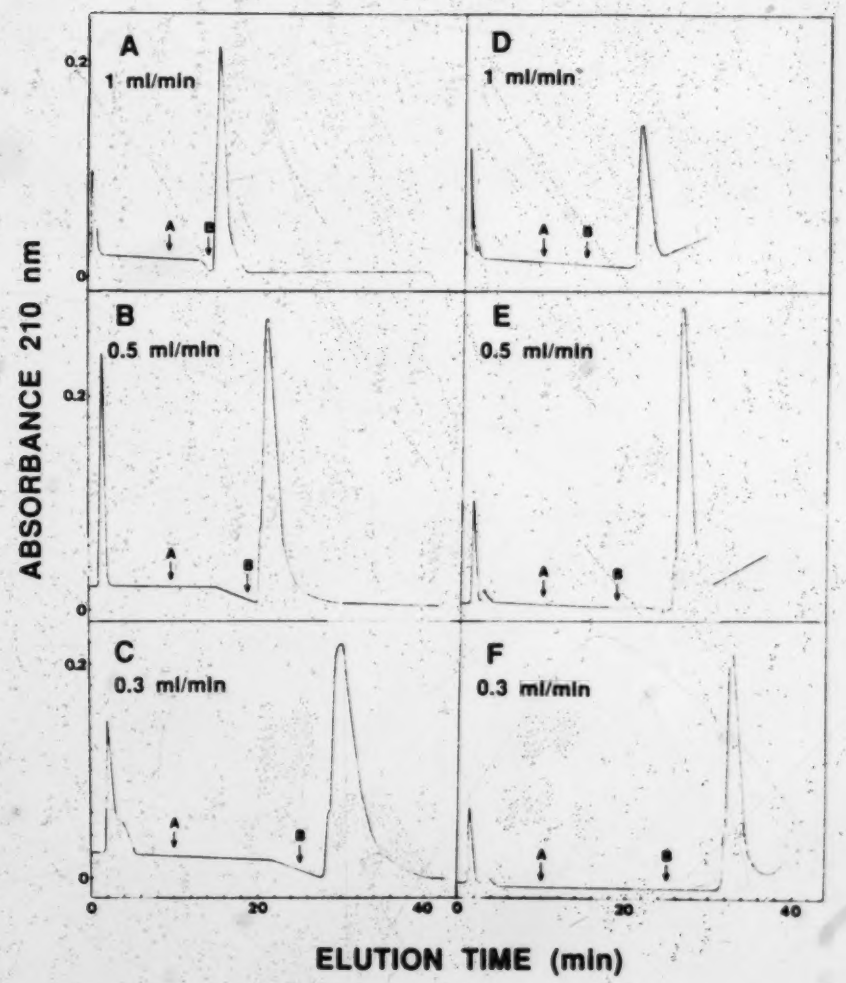

Figure 10. The effect of flow-rate on the elution of the troponin I peptide from a troponin C HPAC column. The column, HPLC instrument and mobile phases are described in Figure 9. Panels A-C: step gradient elution (200 mM KCl). Panels D-F: linear gradient elution (20 mM KCl/min). Flow-rate: 1 ml/min (panels A and D), 0.5 ml/min (panels B and E) or 0.3 ml/min (panels C and F). A denotes the gradient delay time, t_d (described in the text); B denotes the start of the linear gradient or step gradient KCl front, following a 10-min delay (B minus A) after sample application (5 nmole peptide).

Table 4. Effect of Run Conditions on Chromatographic Parameters - Peptide Elution from Protein HPAC Column

Step Gradient Elution

Panel[a]	Flow-rate (ml/min)	t_r[b] (min)	Buffer B[c] ionic strength (mM KCl)	$W_{1/2}$[d] (min)	Peak height (mAU)	Peak Elution[e] volume (ml)
A	1	17	200	0.8	220	0.80
B	0.5	22.5	200	1.6	272	0.80
C	0.3	30.0	200	2.9	224	0.87
-	0.3*	25.7	600	0.8	600	0.24
-	0.3*	26.7	400	1.0	487	0.31
-	0.3*	32.0	200	2.05	200	0.62
Linear Gradient Elution			(mM/min)			
D	1	21.5	20	1.4	133	1.40
E	0.5	26.8	20	1.4	276	0.70
F	0.3	33.2	20	1.4	224	0.42
-	0.3*	37.2	15	1.2	259	0.36
-	0.3*	45.8	10	2.7	160	0.81
-	0.3*	61.3	5	4.5	103	1.35

[a] Refers to panels in Figure 10.

[b] Denotes retention time; all runs had an initial 10-min delay time following sample application and prior to the start of the linear gradient or step gradient KCl front.

[c] The desorbing buffer during step gradient elution.

[d] Peak width at half height.

[e] Peak elution volume is calculated by multiplying $W_{1/2}$ by the flow-rate.

* Runs marked with a star were carried out on a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.), coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive HP2225A Thinkjet printer and HP7440A plotter. The unmarked runs were carried out on a Vista Series 5000 LC coupled to a Schoeffel GM770 single wavelength detector and Technical Marketing plotter.

width remained constant with varying flow-rate during linear gradient elution, but increased with decreasing flow-rate during step gradient elution; there was a general increase in peak height with decreasing flow-rate in both elution modes; finally, peak elution volume decreased with decreasing flow-rate during linear gradient elution, but remained essentially constant during step gradient elution. It should be noted that, when a protein, troponin T (TnT; MW=30,500 daltons), was used as the solute molecule on the TnC HPAC column, there was a small increase in peak width at low flow-rates during linear gradient elution. This is in contrast to the results shown in Table 4 for the TnI peptide, where peak width was unaffected by flow-rate variations during linear gradient elution. This increase in peak width observed for TnT was most likely due to enhanced diffusion of the protein at lower flow-rates. With either a peptide or protein as the solute molecule, peak heights are larger at slower flow-rates because the eluted solute molecule spends more time in the detector than at faster rates, i.e., the detector has more time to "see" the molecule at slower flow-rates. The constant elution volume, with varying flow-rate, of the peptide during step gradient elution, indicated that peak width increased in direct proportion to the decrease in flow-rate. These effect of varying flow rate on peptide elution profiles have been previously documented (Burke *et al.*, 1991).

Table 4 also reports the effect on peptide characteristics of elution at constant flow-rate, but varying gradient-rate during linear gradient elution, or varying ionic strength of the desorbing buffer during step gradient elution. Thus, peptide retention time increased with both decreasing gradient-rate and decreasing ionic strength of the desorbing buffer, although the latter decrease was not as dramatic as the former; peak width increased with both decreasing gradient-rate and decreasing ionic strength of the desorbing buffer; peak height decreased with both decreasing gradient-rate and decreasing ionic strength of the desorbing buffer; finally, as expected from the peak width results, peak elution volume

increased with both decreasing gradient-rate and decreasing ionic strength of the desorbing buffer.

The relationship between dynamic sample capacity (capacity under flow) and flow-rate depends on both the diffusion rate of the solute and the chemical association rate of the solute/ligand interaction (Technical brochure from Beckman Inst.). When maximum capacity is required, the flow-rate can be decreased until the dynamic capacity approaches the equilibrium capacity. On an HPAC column, this point is reached at a higher linear velocity than for conventional soft gel columns. This is because the mass transfer rate possible with the small particles of HPAC supports is much higher than that for large particles. As a result, HPAC packings allow more efficient use of all the immobilized ligand at any flow-rate. Thus, at higher flow-rates, the dynamic capacity of the Ultraffinity-EP columns (10-μm particle size) used in the present study is typically one order of magnitude higher than conventional (100-μm particle size) affinity support material. This results from a combination of the higher mass transfer rate of the solute into and out of the affinity matrix and the high ligand concentration possible. It is only at extremely high flow-rates that the capacity of an HPAC column decreases to any degree. At these high flow-rates, the back pressure is great, perhaps damaging the ligand, and should not be used. An average HPAC column can be successfully used at a linear flow rate of 3.0-6.0 cm/min (equivalent to a flow-rate of 0.5-1.0 ml/min on an analytical column).

CARE AND MAINTENANCE OF COLUMNS

After derivatization of the support, the HPAC column should be stored in a low salt buffer with 0.02% bactericide (NaN_3) at 4°C. The column should not be frozen. Also, care must be taken to ensure that the pH of the storage buffer does not go above pH 7 if the column support is silica. It is extremely important to use preinjection filters and in-line filters to minimize plugging of the column. The stability and lifetime of HPAC columns may be extensive, as long as care is taken with them.

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CHAPTER 4

CALMODULIN AND TROPONIN C: AFFINITY CHROMATOGRAPHIC STUDY OF DIVALENT CATION REQUIREMENTS FOR TROPONIN I INHIBITORY PEPTIDE (104-115), MASTOPARAN, AND FLUPHENAZINE BINDING

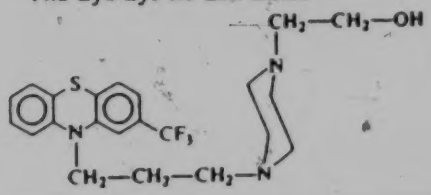
INTRODUCTION

Since muscle contraction is triggered by the binding of Ca^{2+} to low affinity sites of TnC, it is essential to expand our understanding of the molecular events involved in the transmission of the Ca^{2+} signal. In particular, it is important to determine the cation specificity of the interactions between TnC and several of its target molecules. The structural changes produced by binding of Mg^{2+} or Ca^{2+} to TnC and its related molecule, calmodulin (CaM), results in the exposure of various binding interfaces capable of different interactions with target molecules (LaPorte *et al.*, 1980; Gariépy and Hodges, 1983a; Cachia *et al.*, 1985). In this chapter, the cation requirement (Mg^{2+} or Ca^{2+}) for TnC or CaM binding to three different target molecules: the synthetic TnI peptide 104-115 (which mimics TnI), a wasp venom peptide (mastoparan) and a phenothiazine drug (fluphenazine) is determined. These target molecules have been assumed to mimic naturally occurring binding sites of TnC- and CaM-binding proteins. This work is published in Biochem. Cell Biol. **65**:982-988 (1988).

The homology between the CaM and TnC primary sequences are reflected in the large degree of similarity in the tertiary structures of the proteins. Both molecules have two major folded domains, each containing two Ca^{2+}-binding sites separated by a long central helix (Herzberg and James, 1985; Sundaralingam *et al.*, 1985; Babu *et al.* 1985). TnC has two classes of Ca^{2+} binding sites. The low affinity Ca^{2+}-specific sites (I and II) are

located in the N-terminal region of the molecule, while the high affinity sites (III and IV) which bind Ca^{2+} and Mg^{2+} ions competitively are located in the C-terminal domain. CaM has four binding sites which have association constants for Ca^{2+} falling within one order of magnitude of one another. It is generally accepted that the four Ca^{2+}-specific sites of CaM can be divided into two subclasses: sites I and II with lower affinities and sites III and IV having higher affinities (Anderson *et al.*, 1983; Aulabaugh *et al.*, 1984; Klevit *et al.* 1984). Ilda and Potter (1986) concluded that CaM has four equivalent Ca^{2+}-binding sites, which compete for both Mg^{2+} and Ca^{2+}. The difference in the interactions of the two proteins with Mg^{2+} is evident in the lower Mg^{2+} association constant for CaM of 130 M^{-1} (Ogawa and Tanokura, 1984) compared with 5000 M^{-1} for TnC (Potter and Gergely, 1975). As well, the conformational change induced upon Mg^{2+} binding is much reduced in CaM as compared with TnC (Tsalkova and Privalov, 1985), mainly because CaM has more secondary structure than TnC in the absence of metal ions.

Table 5. Structure of ligands bound to the affinity columns

Ligand	Structure
TnI inhibitory peptide 104–115[a]	Ac-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-amide
Mastoparan[b]	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-amide
Fluphenazine	

[a] Sequence from Cachia et al., 1983.
[b] Sequence from Malencik and Anderson, 1983.

Recent work has focused on determining the common features that enable different peptides and drugs to bind to TnC and CaM (for review see Cachia *et al.*, 1985). Our laboratory has drawn attention to the differences in conformation adopted by mastoparan and the TnI peptide 104-115 on binding to TnC and CaM (Cachia *et al.*, 1986). These results have cast a significant degree of doubt on the suitability of using only basic amphiphilic helical compounds, such as mastoparan as a model for CaM-enzyme interactions. There appears to be no related secondary structural requirements in the peptides for their binding to TnC or CaM (Cachia *et al.*, 1986), but the presence of basic charged groups, as well as hydrophobic areas, are important to both peptide (Cachia *et al.*, 1986; Van Eyk and Hodges, 1986) and phenothiazine binding (Weiss *et al.*, 1974; Levin and Weiss, 1978). By analogy, the receptor site on TnC and CaM requires the specific and spatially correct alignment of oppositely charged amino acids and hydrophobic groups to ensure interaction with each ligand. Since the structure of TnC and CaM differs depending whether Mg^{2+} or Ca^{2+} is bound to the protein, the binding interface for these target molecules may also be dependent on which cation is bound and hence, could dictate whether complex formation occurs between TnC and various target molecules. This study demonstrates that the TnI peptide binds to TnC and CaM in the presence of either Ca^{2+} or Mg^{2+} while mastoparan and fluphenazine behave differently. Therefore, the interface requirements for binding of mastoparan and fluphenazine are such that they may not reflect accurately the physiological interactions of TnC and CaM with their target proteins.

RESULTS AND DISCUSSION

The synthetic TnI peptide 104-115 (Table 5) contains the minimum sequence required to produce inhibition of actomyosin ATPase activity (Talbot and Hodges, 1981). TnC binds to this peptide, causing the partial release of inhibition in the presence of Mg^{2+} and full release upon addition of Ca^{2+} (Cachia *et al.*, 1986; Van Eyk and Hodges, 1988).

There has been much speculation concerning which one of the three regions of TnC that interacts with TnI binds the TnI inhibitory region (Cachia *et al.*, 1985). Enzymatic fragments of TnC and CaM have been utilized to determine which region of these proteins is capable of interacting with TnI and can substitute for TnC in neutralizing the TnI-mediated inhibition of the actomyosin ATPase activity (Walsh *et al.*, 1977; Kuznicki *et al.*, 1981; Weeks and Perry, 1978; Wall *et al.*, 1981). The results of these studies implicate the fragments that contained residues 78-106 of CaM and the analogous region of TnC, residues 84-115, as having both properties. Interestingly, all the active fragments contain the N-terminal α-helix of site III(III_N helix = helix E). The involvement of helix E is supported by fluorescent and photoaffinity labelling results, showing Cys 98 of TnC is in close proximity to TnI (Leavis *et al.*, 1978; Leavis and Tao, 1980; Chong and Hodges, 1981). As well, spectroscopic studies have indicated that TnI peptide 104-115 binding to Ca^{2+}-free TnC results in TnC undergoing helix formation (Cachia *et al.*, 1986), which is similar to the III_N helix formation observed upon Ca^{2+} binding (Leavis *et al.*, 1978). Synthetic peptide studies of Ca^{2+}-binding site III showed that upon Ca^{2+} binding the N-terminal helix (III_N = helix E) is induced (Reid *et al.*, 1981).

This study clearly demonstrates that TnC interacts strongly with the TnI peptide 104-115 affinity column when Mg^{2+} or Ca^{2+} is present, but not in their absence (Table 6). This is consistent with the finding that the interaction between TnC and TnI in Mg^{2+} is stronger by two orders of magnitude than in EDTA and only one order of magnitude weaker than in Ca^{2+} (Ingraham and Swenson, 1984). Since TnI peptide 104-115 requires the presence of Mg^{2+} or Ca^{2+} to interact, the TnI peptide cannot be interacting with site I or II, the Ca^{2+}-specific sites, or site IV, which binds TnI even in the absence of cations. Our results are consistent with the suggestion that helix E of TnC interacts with TnI region 104-115. CaM binding of the TnI peptide 104-115 requires the presence of Mg^{2+} or Ca^{2+} (Table 6). This dependence on Mg^{2+}, as well as on Ca^{2+}, had been suggested previously

in our laboratory by the fact that CaM in the presence of Mg^{2+} produces partial release of the inhibition of the ATPase activity by TnI peptide, with further release in the presence of Ca^{2+} (Cachia *et al.*, 1986). The Mg^{2+} sensitivity of the CaM - TnI 104-115 interaction contradicts earlier actomyosin ATPase studies of Amphlett *et al.* (1976) and Castellani *et al.* (1980). Amphlett reported that CaM, unlike TnC, could neutralize TnI inhibition only in the presence of Ca^{2+} and not in the presence of Mg^{2+}. However, the conditions of their ATPase assays were such that there were equimolar amounts of ATP and $MgCl_2$. Under these conditions the majority of Mg^{2+} is complexed with ATP, leaving virtually no Mg^{2+} free to bind to CaM (Storer and Cornish-Bowden, 1976). Castellani demonstrated that TnC bound TnI in low and high Ca^{2+} concentrations, while CaM was associated with TnI only in high Ca^{2+} conditions (Castellani *et al.*, 1980). The binding experiments were carried out in 1 mM ATP and 2 mM Mg^{2+}, which means there is approximately 1 mM Mg^{2+} in solution to complex with the proteins. Owing to the large differences in Mg^{2+} association constants, under these conditions (130 and 5000 M^{-1} for CaM and TnC, respectively) TnC has all possible sites filled (1.6 Mg^{2+} sites filled out of two sites), while CaM will have only one half of a site filled with Mg^{2+} (0.46 out of four possible sites). The studies of both Amphlett *et al.* (1976) and Castellani *et al.* (1980) are actually monitoring the action of CaM only in the absence of cations (Mg^{2+} and Ca^{2+}) and in the presence of saturating Ca^{2+}. Therefore, with these considerations, both experiments would agree with our results that TnC and CaM interact with TnI sequence 104-115 in the presence of Mg^{2+}, as well as Ca^{2+}, but not in the absence of the divalent cations.

TnI inhibitory peptide has the same divalent binding requirements for both TnC and CaM. This implies that the binding interfaces (exposed in the present of Mg^{2+} or Ca^{2+}) contain similar spatially oriented residues needed for the peptide to bind. This is supported

Table 6. Binding of CaM and TnC to affinity columns

Ligand	Sample applied	First Buffer[a] Buffer variant	First Buffer[a] Binding[c]	Second Buffer[b] [EDTA], M	Second Buffer[b] Binding[c]
TnI peptide 104-115	TnC	EDTA	Yes	0.1	---
		Mg^{2+}	---		Yes
		Ca^{2+}	---		Yes
	CaM	EDTA	Yes		---
		Mg^{2+}	---		Yes
		Ca^{2+}	---		Yes
Mastoparan	TnC	EDTA	Yes	0.1	---
		Mg^{2+}	Yes		---
		Ca^{2+}	---		Yes
	CaM	EDTA	Yes		---
		Mg^{2+}	Yes		Yes
		Ca^{2+}	---		Yes
Fluphenazine	TnC	EDTA	Yes	0.01	---
		Mg^{2+}	Yes		---
		Ca^{2+}	---		Yes
	CaM	EDTA	Yes		---
		Mg^{2+}	Yes		---
		Ca^{2+}	---		Yes

a The first buffer contained 10 mM Mops; 100 mM NaCl; and 0.1 mM EDTA (pH 7.2), 5 mM $MgCl_2$, or 2 mM $CaCl_2$.

b The second buffer contained 10 mM Mops, 100 mM NaCl (pH 7.2), and either 0.1 or 0.01 M EDTA.

c Yes denotes that the protein was eluted from the affinity column using the elution buffer, --- denotes that the protein was not eluted either because it was still bound to the affinity column or else it had been previously eluted from the column.

by the high sequence homology between the two protein's III_N helix that offer hydrophobic and negative-charged regions for binding.

In addition to TnI inhibitory peptide, there are a variety of other natural and synthetic peptides that can modify the actions of CaM and TnC (Cachia *et al.*, 1985). Malencik and Anderson (1982) have proposed that the interactions of these peptides with TnC and CaM reflect similarities between the peptides and parts of the recognition sequence of the TnC- or CaM-binding target proteins. The mastoparans, a group of cytotoxic peptides have exceptionally high affinities for both TnC and CaM (Malencik and Anderson, 1983). Mastoparan (Table 5), a tetradecapeptide from the Vespidae wasp, has the smallest known dissociation constant (K_d = 0.3 nM) of any known peptide or protein which binds to CaM (Malencik and Anderson, 1983). As a consequence, it has been employed often as a model system for studying protein interactions with CaM. Mastoparan X and Polistes mastoparan are known to form basic amphiphilic α-helices upon binding to the hydrophobic surface of CaM (McDowell *et al.*, 1985), while mastoparan has been shown to do so upon binding to both CaM and TnC in the presence of Ca^{2+} (Cachia *et al.*, 1986). It has been hypothesized by Cox *et al.* (1985) that a basic amphiphilic α-helix of 2.8 helical turns in length is the minimum structural requirement for binding. However, Cachia *et al.* (1986) demonstrated that TnI peptide 104-115 does not exhibit any structure in solution even in the presence of helix-inducing solvents (see Chapter 6 and 7, Rüegg et al., 1989; Van Eyk et al., 1991). Our results further confirm that TnI peptide 104-115 and mastoparan do not have the same protein interface binding requirements. NMR results suggest that both peptides bind at the same region of TnC (P.J. Cachia, J. Gariépy, and R.S. Hodges, unpublished results). CaM and TnC form Ca^{2+}-dependent complexes with a mastoparan affinity column (Table 6 , Fig. 11). Both proteins are eluted with EDTA; however, TnC is retained longer on the column (29 versus 6.4 ml) than CaM. This effect

can not be totally explained by the differences in the Ca^{2+} off-rates of the two proteins (1 s^{-1} for the high affinity sites of TnC and 50 s^{-1} for the high affinity sites of CaM).

In the presence of Mg^{2+}, CaM was partially bound to a mastoparan affinity column while TnC was not (Fig. 11). The CaM-mastoparan interaction is weak with approximately one tenth of the possible binding occurring. This interaction is important since CaM, but not TnC, is capable of partially releasing the mastoparan-induced inhibition of the acto-S1 ATPase activity (Cachia *et al.*, 1986). In the presence of Ca^{2+}, both proteins release the mastoparan inhibition. In addition, a circular dichroism study indicates in the absence of divalent cations, TnC and CaM form complexes with mastoparan (Cachia *et al.*, 1986). These differences in cation requirements for mastoparan interaction with TnC and CaM imply that these proteins have different conformations at the ligand binding site depending on which cation is present. This supports the observation that Mg^{2+} potentiates Tn inhibition of the acto-S1 ATPase, while Ca^{2+} potentiates the release of inhibition (Van Eyk *et al.*, 1986). The difference in the interactions of TnC and CaM with mastoparan in the presence of Mg^{2+} is likely due either to the interaction between mastoparan and TnC (Mg^{2+}) being very weak and therefore could not be monitored by affinity chromatography or the binding site for mastoparan is not exposed in TnC unless Ca^{2+} versus Mg^{2+} is bound to sites III and IV.

The phenothiazine antipsychotic drugs are among the most extensively studied group of CaM antagonists. The phenothiazine binding causes inhibition of the CaM activation of phosphodiesterase and the Ca^{2+}-dependent erythrocyte Ca^{2+},Mg^{2+}-ATPase (Levin & Weiss, 1979; Roufogalis, 1981). There are two distinct classes of phenothiazine binding sites on CaM: a class of high affinity Ca^{2+}-dependent sites and a class of low affinity Ca^{2+}-independent sites. It is the former that is thought to be physiologically important for binding target enzymes (Levin and Weiss, 1979; Weiss *et al.*, 1980). TFP (a phenothiazine drug related to the drug employed in this study, Table 5), like TnI peptide and mastoparan, has a binding site at the III_N helix of TnC (Gariépy and Hodges, 1983b).

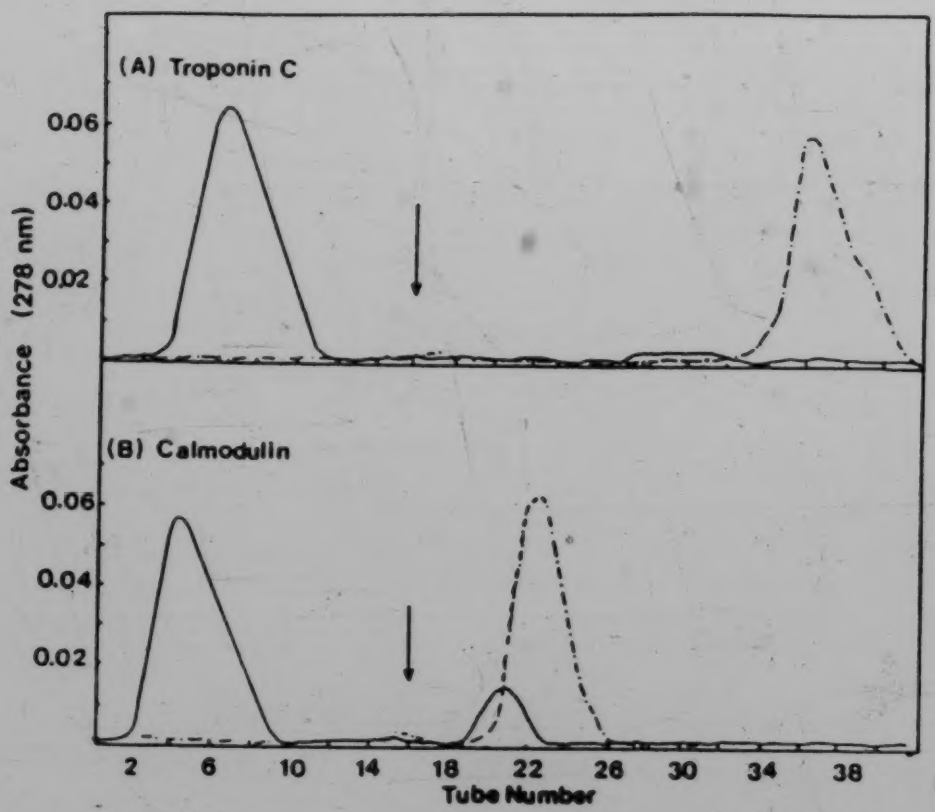


Figure 11: The difference of Mg^{2+} or Ca^{2+} on the binding of TnC or CaM to a mastoparan-Sepharose affinity column. (A) Profile of TnC in Mg^{2+} (—) or Ca^{2+} (-··-) buffer. (B) Profile of CaM in Mg^{2+} (—) or Ca^{2+} (-··-) buffer. Application of 0.1M EDTA buffer to column is indicated by solid arrow.

The binding of TnC and CaM to a fluphenazine affinity column was Ca^{2+} dependent (Table 6). The fluphenazine-protein interactions were the weakest studied, with TnC and CaM eluting in 10 mM instead of 100 mM EDTA. The binding of fluphenazine to TnC or CaM does increase the cation-binding constants, since 1 mM EDTA is sufficient to remove Ca^{2+} from TnC or CaM when the peptides or fluphenazine are absent. This concurs with the dramatic increase in the extent of Ca^{2+} binding when trifluoroperazine, a related

phenothiazine drug, is bound to CaM (Keller *et al.*, 1982; Malencik and Anderson, 1984). All three ligands increase the Ca^{2+}- and Mg^{2+}-binding affinities when complexed to TnC or CaM.

It is the α-helices surrounding the Ca^{2+}-binding loops that stabilize cation binding (Chong and Hodges, 1981; Gariépy *et al.*, 1982). The majority of evidence the indicates that the three ligands examined in this study all bind near helix E of TnC or the homologous region in CaM, suggesting that the compounds influence the stability of that helix and hence the cation-binding constants. Phenothiazines are known to increase the α-helical content of CaM when bound and the quantity of structural change induced is related to the *in vitro* ability of the drug to inhibit CaM-dependent phosphodiesterase (Levin and Weiss, 1979; Prozialeck and Weiss, 1982). Mastoparan increases the α-helical content of TnC, while TnI peptide and mastoparan produced minor changes in CaM upon binding (Cachia *et al.*, 1986). The elution of TnC or CaM from the TnI inhibitory peptide affinity column requires a 10-fold larger concentration of EDTA than that required for elution from a fluphenazine affinity column. This implies that TnI inhibitory peptide stabilizes the Ca^{2+} binding more effectively than does the drug. There is an increase in the TnI (residues 104-115) binding constant for TnC in the presence of Ca^{2+} compared with Mg^{2+} (Van Eyk and Hodges, 1988). The three ligands all influence the cation binding to TnC and CaM, emphasizing the importance of the tertiary structures of the Ca^{2+}-binding units.

Neither TnC nor CaM binds to the fluphenazine column in the presence of Mg^{2+}. This is interesting, since TnC will not bind at a Ca^{2+} concentration in which the Ca^{2+}-Mg^{2+} sites are not filled. At a Ca^{2+} concentration of 5×10^{-6} M where the high affinity sites are filled, TnC binds and can be eluted from a fluphenazine column (P.J. Cachia, J. Gariépy, and R.S. Hodges, unpublished results). It appears that fluphenazine requires the Ca^{2+}-induced conformation of site III to bind to TnC rather than that induced by Mg^{2+}. The differences between the Ca^{2+} and Mg^{2+} conformers of TnC and CaM appear to be

enough to alter the spatial orientation of the binding interface and influence the binding of the three ligands.

RECENT DEVELOPMENTS

Since TnI 104-115 required the presence of Mg^{2+} or Ca^{2+} to interact with TnC or CaM, it was suggested that the TnI peptide cannot be interacting with TnC sites I or II, the Ca^{2+}-specific sites or site IV, which binds TnI even in the absence of cations. Therefore, the TnI peptide must be interacting near the Ca^{2+} binding site III (for review see Cachia *et al.*, 1983). Since publication of this paper, other research has continued to indicate that the TnI residues 104-115 interacts with C-terminal domain of TnC, in particular residues 89 to 100 (the residues that compose helix E, the N-terminal helix of Ca^{2+} binding site III). The most convincing evidence for the interaction between TnI and residues 89 to 100 of TnC has been provided by photo-crosslinking experiments (Lesyzk *et al.*, 1987, 1988) in which Cys 98 of TnC was modified with a benzophenone photo-crosslinker. It was clearly shown that the inhibitory region of TnI was close to Cys 98 of TnC in both the IC and Tn complex. On the other hand, there is some evidence that the TnI inhibitory region can also interact with the N-terminal domain of TnC. The zero-length crosslinker, EDC, crosslinked the TnI inhibitory region (residues Lys 105 and 107) to the N-terminus of TnC (residues 46-78) (Lesyzk et al., 1990). It is important to note that EDC is very specific requiring amino groups within crosslinking distances. This could bias the results and select for a reduced number of TnI-TnC interactions due to distance constraints or intramolecular crosslinking. This has apparently occurred since there was no crosslinking between the N-terminal segment of TnI (residues 1-22) and TnC (Syska *et al.*, 1976). TnI residues 1-22 contains two Lys (residues 5 and 18) but these Lys have been shown not to be involved in the TnI-TnC interaction. Regardless of the limitations of EDC as a crosslinking reagent, the work suggests there may be an interaction between TnI, residues

104-115 and N-terminal domain of TnC, residues 46-78. However as the authors state this does not negate the importance of the interaction between the TnI inhibitory region and C-terminal domain of TnC. As well, Lan *et al.* (1990) indicated that the preferred binding site of the synthetic peptide is on the C-terminal domain of CaM or TnC. In fact, the TnI peptide did not alter the fluorescence emanating from a probe on Met 25 but did alter the environment around Cys 98 (further discussion in Chapter 7).

A phenothiazine drug which is related to fluphenazine, TFP has been shown to bind to both domains of skeletal TnC in the presence of saturating Ca^{2+} (Klevit *et al.*,1981, Forsén *et al.*, 1980, Krebs and Carafoli, 1982, Massom *et al.*, 1990). Modelling of TFP binding to CaM and TnC (Strynadka and James, 1988) suggested that TFP could bind to hydrophobic pockets in both the C- and N-terminal domains of TnC or CaM. The two domains of TnC and CaM are cup-shaped and the interior residues of the cup are hydrophobic and form a pocket. The amino acids located on the outside and rim of the cup are generally charged residues. Residues from helix E (residues 89-100) of TnC and equivalent helix in CaM (residues 81-93) compose one side of this cup. Therefore, molecules which bind in the hydrophobic pocket would potentially interact with helix E. In the presence of Mg^{2+} or Ca^{2+}, the hydrophobic pocket in the C-terminal domain is available for interaction with target molecule, but as this and other studies have indicated the binding interface in the C-terminal domain differs depending on which cation is bound. Gariepy and Hodges (1983b) showed TFP could bind to synthetic peptide of helix E as well as a proteolytic fragment composed of helix-loop-helix of Ca^{2+} binding site III. Recent work showed that TFP only bound to TnC when the Ca^{2+} binding site II was filled (Massom *et al.*,1990). This is important since the hydrophobic pocket located in the N-terminal domain of TnC is available only when Ca^{2+} is bound to sites I and II. Several solution studies have implicated the movement of hydrophobic residues in the N-terminal domain to a more solvent exposed state upon Ca^{2+} binding (Levine et al.,1977; Cachia et

al., 1985; Drabikowski et al., 1985). In contrast, binding of Mg^{2+} to sites III and IV causes the formation of the antiparallel β sheets in both the C- and N-domains of TnC, but changes to the hydrophobic pocket only occurs in the C-terminal domain with no detectable change in the N-terminal domain (Tsudu *et al.*,1988, 1990).

These results taken together suggest that in the absence of Ca^{2+}, when sites III and IV are filled, the primary binding site for TnI peptide and other TnC binding molecules is located in the C-terminal domain. However, in the presence of saturating Ca^{2+}, the associated conformational change in TnC opens a previously closed hydrophobic pocket located in the N-terminal domain to which these molecules can possibly bind. The physiological relevance of the possible interaction by the inhibitory region of TnI with the N-terminal domain of TnC is unknown. Residues 46-78 of TnC (to which TnI was linked by EDC, and where TFP and melittin are proposed to bind) comprises part of the Ca^{2+}-binding site II which is one of the regulatory Ca^{2+} binding sites. Neither this TnC fragment nor a fragment of the entire N-terminal domain (residues 9-84) can mimic the activating properties of the whole TnC molecule (Grabarek *et al.*, 1981). A slightly longer fragment containing residues 1-100 had approximately 30% activity of the intact molecule when substituted for TnC in the actomyosin ATPase assay, while TnC fragment (residues 89 to 159) had approximately 70% of the activity of native TnC.

Whether TnI peptide or mastoparan can bind to both domains simultaneously like melittin is debatable (Seeholzer *et al.*,1986). Melittin is twice the length of the TnI peptide or mastoparan, which allows it to span the length of the central helix which separates the two domains of TnC and CaM. Unless the central helix (D/E linker) can kink, bringing the two domains in close proximity, it seems physically impossible for a single TnI peptide to bind simultaneously to both domains of TnC. There is some evidence under more physiological conditions than those under which TnC was crystallized (Herzberg and James, 1985, Sundaralingam *et al.*, 1985), that TnC is more compact and not an extended

molecule (Hubbard *et al.*, 1988, Heidorn and Trewhella, 1988, Wang *et al.*, 1987, Fujisawa *et al.*, 1989). However, this debate over the flexibility and function of the central helix remains unsolved for the present time.

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CHAPTER 5

THE BIOLOGICAL IMPORTANCE OF EACH AMINO ACID RESIDUE OF THE TROPONIN I INHIBITORY SEQUENCE 104-115 IN THE INTERACTION WITH TROPONIN C AND TROPOMYOSIN-ACTIN

INTRODUCTION

The synthetic TnI peptide 104-115 mimics TnI by inhibiting the acto-S1 ATPase activity, possessing TM specificity and binding TnC which results in the release of the ATPase inhibition. Thus the TnI sequence 104-115 is responsible in part for the Ca^{2+}-sensitive control exhibited by Tn. Since the minimum sequence required for inhibition has been clearly established to be residues 104-115, the next level of complexity is to determine the contribution of each amino acid residue of the TnI inhibitory region toward the interaction with TM-actin and TnC. In this chapter, a series of synthetic analogs of the TnI inhibitory region in which one or more amino acid residue(s) was substituted by glycine were quantitatively compared by monitoring both the extent of inhibition of the ATPase activity induced by the analogs and the strength of binding between the analogs and TnC in the presence of Mg^{2+} or Ca^{2+}. This work published in 1988 in the Journal of Biological Chemistry **263,** 1726-1732 provides insight into the molecular interactions between the TnI inhibitory region and its target proteins.

RESULTS

INHIBITORY ACTION OF TnI SYNTHETIC ANALOGS ON THE ACTO-S1 ATPase

Synthetic analogs of this sequence in which glycine replaces individual amino acids provide a feasible means of systematically investigating the contribution of each amino

Table 7. Amino Acid Sequence of TnI Inhibitory Peptide 104-115 and Glycine Analogs.
The number(s) designates the analog with residue(s) substituted with glycine(s).
NS denotes the TnI inhibitory peptide of the native sequence (104-115)

Peptide	Sequence
	104 105 106 107 108 109 110 111 112 113 114 115
NS	Ac-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-amide
105	Ac-----Gly----------amide
106	Ac---------Gly----------amide
107	Ac-------------Gly----------amide
108	Ac-----------------Gly----------amide
109	Ac----------------------Gly----------amide
110	Ac-------------------------Gly----------amide
111	Ac-----------------------------Gly----------amide
112	Ac-----------------------------------Gly----------amide
113	Ac---Gly---------amide
114	Ac---Gly-----amide
115	Ac---Gly-amide
105-107	Ac-----Gly-----Gly----------amide
105-107-108	Ac-----Gly-----Gly-Gly----------amide
106-109-110-111-114	Ac---------Gly---------Gly-Gly-Gly---------Gly-----amide

acid side-chain toward the inhibitory mechanism (Table 7). The inhibition of the acto-S1 ATPase activity by the TnI inhibitory peptide (104-115) (denoted as NS) and by the single-glycine substituted analogs is shown in Fig. 12. As previously reported, the TnI inhibitory peptide is an excellent mimic of TnI, possessing 75-92% of the maximum inhibition displayed by TnI. All the TnI analogs inhibited the ATPase activity to varying degrees and were quantitatively less effective than either TnI or the TnI inhibitory peptide.

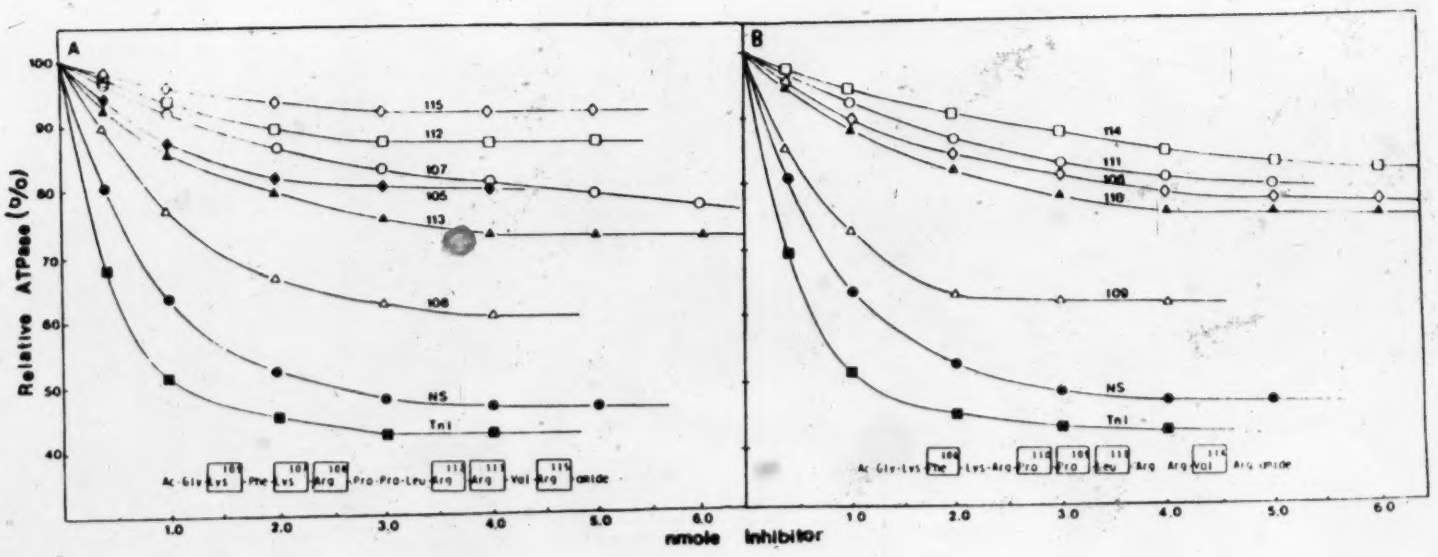

Figure 12. Inhibition of acto-S1-tropomyosin ATPase activity by TnI, TnI inhibitory peptide (104-115) and single glycine-subsituted analogs of this region. S1, actin, and TM concentrations used were 4.3, 1.6 and 0.22 uM, respectively. ATPase activity measurements (2 ml/assay) were carried out using the continuous titration method. The acto-S1-TM ATPase was taken to be 100%. The inhibitory activity of TnI and TnI inhibitory peptide (denoted as NS) was compared with TnI analogs where the basic amino acid residues were substituted with Gly (panel A) or with TnI analogs where non polar amino acids residues were substituted (panel B). The numbers represent the sequence number of the residue substituted with Gly.

The importance of an amino acid side-chain is inversely related to the inhibition produced by the corresponding glycine-substituted analog. The inhibition produced by an analog is a function of the relative binding strengths of the analog and the level of maximum inhibition reached. The latter is a reflection of the capability of the analog to perform the inhibitory action. To evaluate these two parameters, the experimental data for each inhibition curve were plotted as the double reciprocal of % inhibition (1/% inhibition) *versus* inhibitor concentration (1/c). Fig.13 shows a representative double-reciprocal plot of TnI, TnI inhibitory peptide (NS), and analogs Gly-105, Gly-107, Gly-110 and Gly-114. The slope of the resulting linear line is an indication of the ability of the inhibitor to bind to tropomyosin-actin and induce inhibition of the acto-S1 ATPase. The larger the slope value of an analog, the less this analog mimics TnI, and hence, the more important the substituted amino acid is for the functioning of the inhibitory sequence. The amino acid residues of TnI sequence (104-115) are ranked in Table 8, depending on their relative importance.

Two other parameters for each analog, the efficacy factor and C_{50} value, are listed in Table 8. The efficacy factor quantitates the efficiency of an analog to inhibit the acto-S1 ATPase at saturating concentrations of the analog. The closer the efficacy factor of an analog is to the efficacy factor of TnI (65.4), the better this analog is able to mimic TnI inhibition. A high efficacy factor implies that the substituted amino acid does not prevent the analog from inducing the conformational changes in the thin filament necessary to produce inhibition of the ATPase. The C_{50} value is the concentration of inhibitor required to induce 50% of the maximum inhibition. The smaller the C_{50} value for an analog, the greater its affinity for tropomyosin-actin and the less the substituted amino acid is required for binding the TnI inhibitory region to tropomyosin-actin.

A comparison of the TnI and the TnI inhibitory peptide (104-115) values (Table 8) shows approximately equivalent efficacy factors but a 2-fold larger C_{50} value for the peptide. This implies that the TnI inhibitory peptide (104-115) is able to induce the same

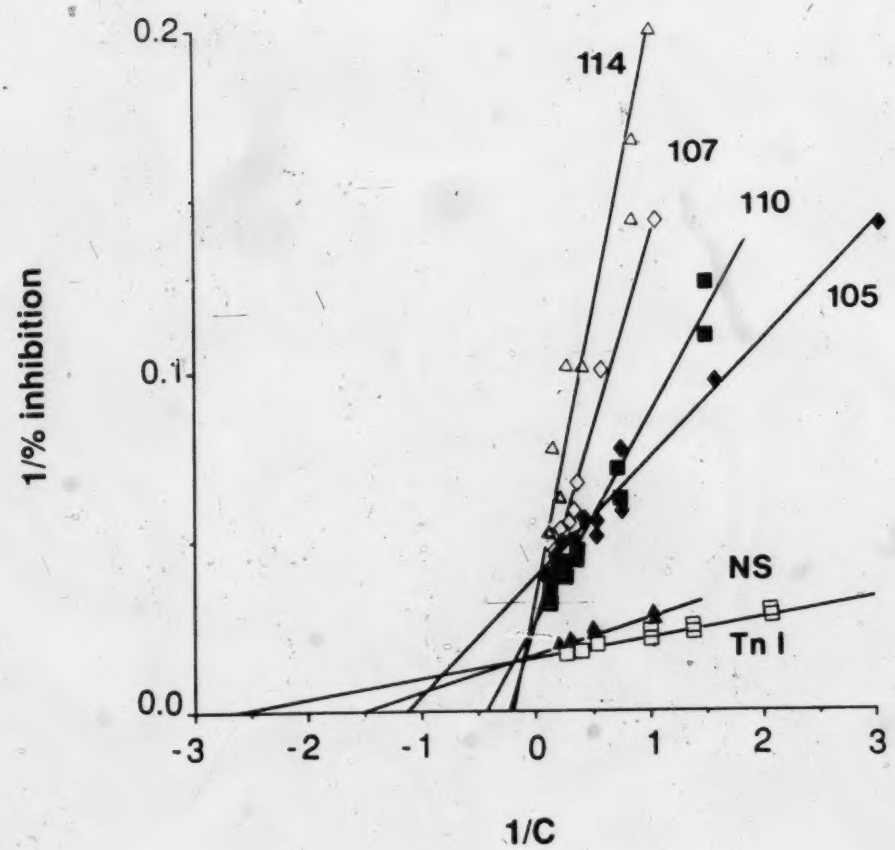

Figure 13. Reciprocal plots of the TM-acto-S1 inhibition by TnI, TnI inhibitory peptide (NS) and analogs Gly-105, Gly-107, Gly-110, and Gly-114 and inhibitor concentration. Experimental data of each inhibitor were plotted as the inverse of % inhibition versus the inverse of inhibitor concentration (nmoles/2 ml assay). Symbols; TnI (□), TnI inhibitory peptide (▲), analogs Gly-105 (♦), Gly-110 (■), Gly-107 (◊) and Gly-114 (△).

Table 8. Evaluation of acto-S1-ATPase inhibition by TnI, TnI inhibitory peptide (NS) and single glycine substituted TnI inhibitory analogs

	Position substituted by Gly	Slope[a]	Efficacy Factor[b]	C_{50} Value[c]
Most essential	Arg-115	0.167	9.2	1.5
residue	Val-114	0.117	21.7	2.5
	Lys-107	0.112	38.2	4.3
	Leu-111	0.103	41.2	4.3
	Arg-112	0.100	20.5	2.0
	Phe-106	0.064	32.7	2.0
	Pro-110	0.060	38.2	2.3
	Arg-113	0.036	31.2	1.1
	Lys-105	0.035	25.5	0.9
Least essential	Arg-108	0.026	52.1	1.3
residue	Pro-109	0.020	52.9	1.0
	NS	0.011	60.6	0.6
	TnI	0.006	65.4	0.3

[a] The slope of the line (equal to C_{50}/EF) is inversely related to the overall ability of the inhibitor to bind to the thin filament and induce the correct conformational changes resulting in the inhibition of the acto-S1-TM ATPase activity.

[b] The efficacy factor determined from the 1/y intercept is the % inhibition of the acto-S1-TM ATPase activity induced by the inhibitor at saturating concentrations of inhibitor. This reflects the efficiency of the inhibitor to inhibit the ATPase activity.

[c] The C_{50} value (nmole inhibitor/assay) determined from the -1/x intercept is the concentration of inhibitor required to induce 50% of the maximum inhibition.

conformations in tropomyosin-actin required for inhibition as TnI, but the peptide binds more weakly to tropomyosin-actin than the native protein. An interesting effect is seen when comparing the analogs Gly-108 and Gly-115. The C_{50} values for these analogs are similar (1.3 and 1.5 nmole/assay, respectively) but the efficacy factors are 52.1 and 9.2, respectively. The reduced efficacy factor for Gly-115 means that the replacement of Arg-115 results in the inability of the remaining residues in the TnI sequence to efficiently produce inhibition, even though the peptide has saturated the tropomyosin-actin binding site. Table 8 shows that every amino acid residue in the TnI sequence (104-115) contributes to the inhibition of the acto-S1 ATPase. However, each residue varies in its importance and effect on the binding and the induction of inhibition.

TnC HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY (HPAC) OF SYNTHETIC TnI ANALOGS IN THE PRESENCE OF Mg^{2+} OR Ca^{2+}.

The ability of the analogs to bind to a TnC affinity column under varying salt conditions determines the contribution of each side-chain of the TnI inhibitory sequence 104-115 toward the interaction with TnC. A TnC-derivatized Beckman Ultraffinity-EP column was utilized to monitor relative binding affinities. Since this method employs HPLC, it provides a quantitatively more accurate means of determining the retention times and concentrations of KCl required to elute the analogs compared to conventional affinity chromatography (methodology review of HPAC see Chapter 3). Three consecutive runs of the native synthetic peptide (104-H5) were eluted within 0.2 min of each other. Since the HPLC runs were finished in 40 min and equilibrated within 20 min, it was possible to examine the binding of many analogs on the same day, eliminating day-to-day fluctuations, a factor which is important when comparing elution times between the various analogs.

Comparison of elution times for the analogs with 1, 2, 3, or 5 glycine substitutions demonstrated that the effect of more than 1 glycine substitution on the retention time of the

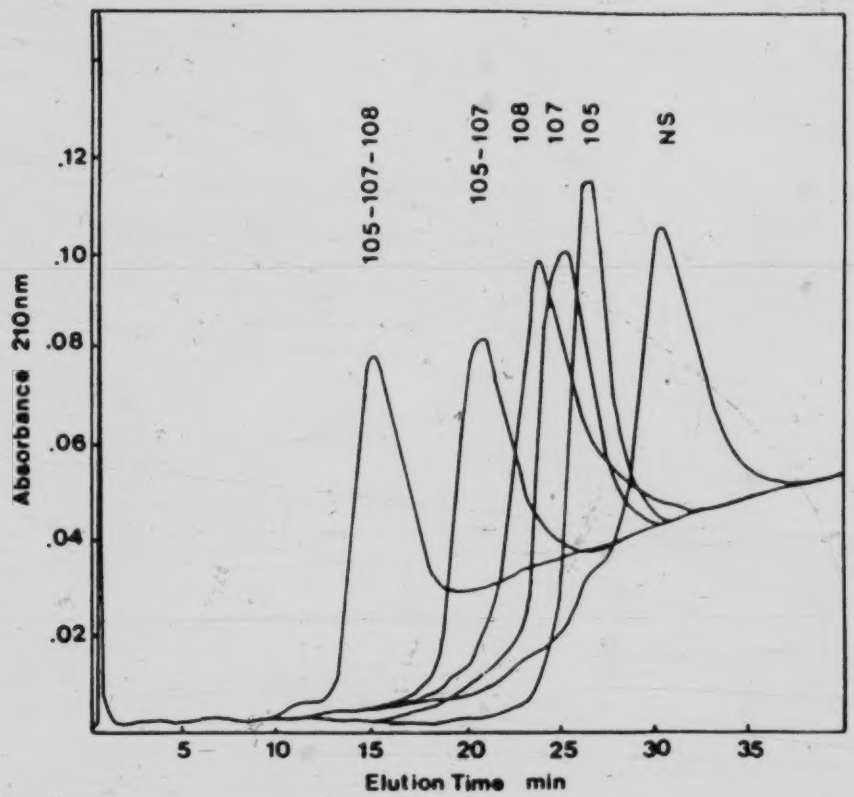

Figure 14. Comparison of retention times of single and multiple glycine-substituted TnI analogs on a HPAC-TnC affinity column in the presence of Ca^{2+}. Individual analogs were chromatographed on an Ultraffinity column derivatized with skeletal TnC. Conditions as stated in Fig. 8. Analogs; single substituted, Gly-105, Gly-107, and Gly-108; doubly substituted, Gly 105-107 and triply substituted, Gly 105-107-108 and NS which is the TnI inhibitory peptide 104-115.

peptide is not the additive effect of the single amino acid replacement (Table 9, Fig. 14). For example, the peptide with 5 glycine residues substituted for the neutral residues leaving all 6 positively-charged residues still present, bound more tightly than expected. It is interesting that, the analogs in which a basic residue was replaced with glycine had narrow peak widths at half-height, compared to the analogs with a neutral or hydrophobic residue substituted with glycine which had significant peak broadening (~2-4 fold).

Table 9 lists the concentration of KCl required to elute each analog from the HPLC TnC-affinity column in the presence of Mg^{2+} or Ca^{2+}. The concentration of KCl required to elute an analog is inversely related to the importance of the substituted amino acid residue in TnC-TnI peptide binding. All analogs were retained on the column but were bound less tightly to TnC than the TnI inhibitory peptide (104-115) (see R_c Mg^{2+} and R_c Ca^{2+}, Table 9). This finding indicates that all of the residues in the sequence play a facilitating role in the interaction with TnC, regardless of which cation, Ca^{2+} or Mg^{2+} was present. The native sequence and all analogs except one (Gly-114) bound more tightly to TnC in the presence of Ca^{2+} than in the presence of Mg^{2+} (compare Rc Mg^{2+} *versus* Rc Ca^{2+}, Table 9). The residues that contribute most to the interaction with TnC in the presence of Mg^{2+} and Ca^{2+} are shown by columns A and B, respectively. The larger the difference between the mM KCl required to elute the native sequence and the analog, the more important is the position that was substituted in the analog. For example, in the presence of Ca^{2+} (column B, Table 9) Phe-106, Leu-111, and Arg-115 which have values of 81, 81 and 94 mM KCl, respectively, are the most essential residues.

As well, in the presence of Ca^{2+}, there is a shift in the interaction toward the C-terminus region of the TnI inhibitory sequence (Table 9, column C). Column C expresses the relative importance of the substituted residue in the presence of Ca^{2+} compared to Mg^{2+}. This is clearly illustrated by analog Gly-114 (Val-114 substituted by glycine) and the TnI inhibitory peptide, which, in the presence of Mg^{2+}, are eluted in 208 and 212 mM

Table 9. Retention time of TnI analog on a TnC affinity column

Position substituted by Gly	Elution conditions: In presence of Mg^{2+}		Elution conditions: In presence of Ca^{2+}		C^c (B-A)
	R_c[a]	A[b]	R_c[a]	B[b]	
	mM KCl		mM KCl		mM KCl
NS[d]	212		249		
Lys-105	143	69	214	35	-34
Phe-106	137	75	168	81	+6
Lys-107	160	52	208	41	-11
Arg-108	144	68	187	62	-6
Pro-109	168	44	219	30	-14
Pro-110	199	13	215	34	+21
Leu-111	157	55	168	81	+26
Arg-112	144	68	178	71	+3
Arg-113	146	66	182	67	+1
Val-114	208	4	178	71	+67
Arg-115	138	74	155	94	+20
105-107			147	102	
105-107-108			88	161	
106-109-110-111-114			129	120	

a Analogs were chromatographed on a Beckman Ultraffinity EP column (4.6 mm ID x 5 cm) derivatized with TnC. Conditions as stated under "Materials and Methods." R_c equals KCl concentration required to elute the peptide (retention time minus tg times the gradient rate of 10 mM KCl/min; for example with NS the retention time in Ca^{2+} was 30.4 min and tg was 5.5 min = 24.9 x 10 = 249 mM).

b The molarity of salt required for elution of the analog was subtracted from the molarity of salt required to elute the TnI inhibitory peptide (NS) in the presence of Mg^{2+} or Ca^{2+}.

c The difference between columns B and A expresses the change in the relative importance of the substituted residue in the presence of Ca^{2+} compared to Mg^{2+}.

d NS denotes the TnI inhibitory peptide (104-115).

KCl, respectively. The difference of 4 mM KCl (column A) in salt concentration required to elute the two peptides indicates that Val 114 is of little importance for binding the TnI inhibitory peptide to TnC in the presence of Mg^{2+}. However, in the presence of Ca^{2+}, the difference of 71 mM KCl (column B) between the elution conditions required to elute the TnI inhibitory peptide (249 mM) and the Gly-114 analog (178 mM) indicates that Val 114 is essential for TnC (Ca^{2+}) interaction. This increased importance of Val 114 in the presence of Ca^{2+} is reflected by +67 mM KCl value (Table 9, column C).

DISCUSSION

Alterations to the amino acid sequence of the TnI inhibitory peptide (104-115) reduce and alter its interactions with tropomyosin-actin and TnC. The importance of each residue toward the functioning of the TnI inhibitory sequence (104-115) is indicated by the extent the analog reduces the interaction with TnC and inhibition of the acto-S1 ATPase activity. All the glycine-substituted analogs inhibit the ATPase activity and are retained on the TnC-affinity column (Tables 8 and 9). This indicates that all amino acid residues in the TnI sequence (104-115) are necessary for full function, with the C-terminal region being most essential for tropomyosin-actin and TnC (Ca^{2+}) interactions. These results correlate well with other less detailed studies. Fig. 15 illustrates the amino acid residues of the TnI inhibitory region determined to be involved in tropomyosin-actin and TnC interactions from this and other studies (Talbot and Hodges, 1981a,b; Dalgarno *et al.*, 1982; Grand *et al.*, 1982; Cachia *et al.*, 1983).

Talbot and Hodges (1979) were the first to use synthetic peptides of the TnI inhibitory region to determine the relationship between the amino acid sequence and the inhibitory potential of this region. Deletion and substituted synthetic peptide analogs of the TnI inhibitory region (104-116) were employed to determine the importance of some selective amino acids. Residues Lys 105 and Val 114 were shown to be essential for

inhibition, while Arg 115 appeared to have little or no effect when deleted from the synthetic peptide. These results are in agreement with our data, with the exception of Arg 115, which our results indicated has a relevant role. The approach used in this study to determine the importance of Arg 115 was to substitute Gly at that position eliminating the contribution of the Arg side-chain toward inhibition but maintaining the peptide backbone. No explanation for this discrepancy can be given. In this study, the sequence of this particular analog (Gly-115) was verified, and multiple assays were repeated on different preparations of acto-S1. The Gly 115 analog was sequenced, digested with trypsin and the fragments were separated by high-voltage paper electrophoresis at pH 6.5 and 1.8. The N-terminus of each fragment was determined by the Dansyl-Edman procedure. The results obtained confirmed that the correct sequence had been synthesized.

Grand *et al.* (1982) employed proton magnetic resonance spectroscopy to identify residues in the sequence of TnI that are involved in the tropomyosin-actin interactions. Only the TnI CNBr fragment CN4 (residues 96-116) displayed a specific interaction with actin. Upon titration of TnI fragment (96-116) with skeletal actin, only Arg residues were perturbed (Fig. 15). However, when whole TnI was titrated, Arg, Leu and/or Val, Lys and Glu residues were perturbed. These differences were rationalized in terms of a conformational constraint on TnI as opposed to the peptide upon interaction with actin. However, the conclusions of Grand *et al.* (1982) are in agreement with this study, i.e., the primary interaction of actin is with the C terminus of the TnI sequence (96-116).

Grand *et al.* (1982) also titrated the CNBr fragments of TnI with TnC in the presence of Ca^{2+}. Specific interactions with TnC were observed with TnI CN5 (residues 1-21) and CN4 (residues 96-116). The Phe, Lys and Leu residues of CN4 (residues 96-116) were perturbed upon addition of TnC (Ca^{2+}), but there was little change in the Arg signal (Fig. 15). Evans and Levine (1980) previously provided evidence that Arg residues were involved in stabilizing the TnI-TnC (Ca^{2+}) complex. As well, Cachia *et al.* (1983),

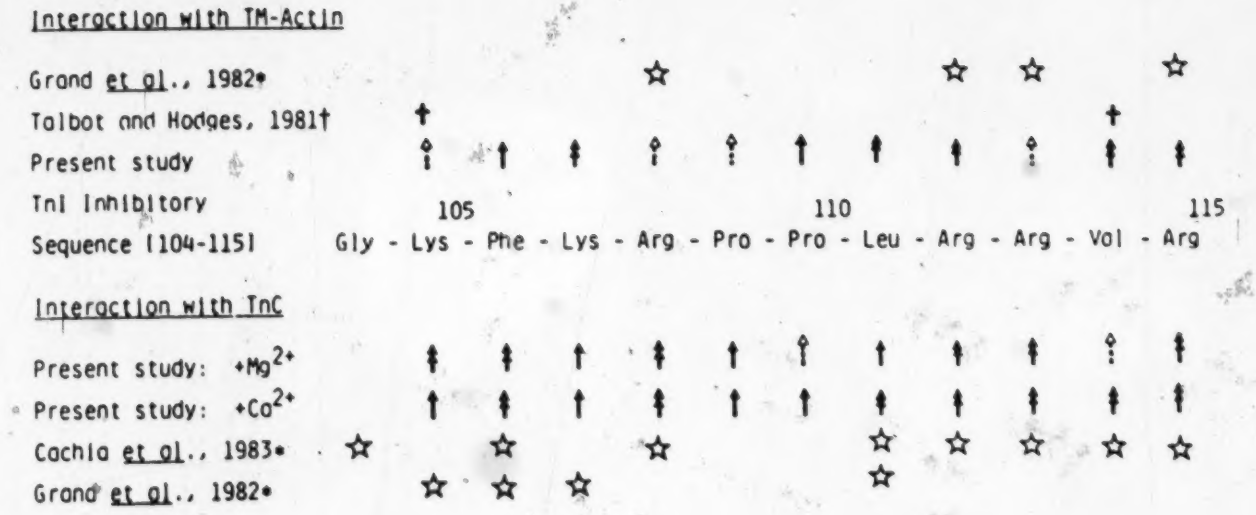
Interaction with TM-Actin
Grand et al., 1982*
Talbot and Hodges, 1981†
Present study
TnI Inhibitory Sequence (104-115)
105
110
115
Gly - Lys - Phe - Lys - Arg - Pro - Pro - Leu - Arg - Arg - Val - Arg
Interaction with TnC
Present study: +Mg2+
Present study: +Ca2+
Cachia et al., 1983*
Grand et al., 1982*

Figure 15. Amino acid residue of TnI inhibitory region (104-115) proposed to be involved in the interaction with TnC and TM-actin. * Data obtained from NMR spectroscopy in the presence of Ca^{2+} and the identification of the specific Arg or Lys residues involved were not determined. Data determined by deletion of the amino acid residues on shortening the peptide chain from either the C- or N-terminal. Double-headed arrows, single-headed arrows, and broken-headed arrows; most ( ), intermediate ( ), and least ( ) essential, determined in this study.

under similar experimental conditions as in the study by Grand, used proton magnetic resonance spectroscopy to investigate the interaction between TnC and the shorter TnI inhibitory peptide (104-115). Leu, Val, Phe and Arg residues of the TnI inhibitory peptide were perturbed upon titration with TnC (Ca^{2+}), while Lys residues showed no involvement (Fig. 15). The differences between the studies by Cachia and that by Grand can be explained in view of the limitations of proton magnetic resonance spectroscopy. This technique is unable to differentiate between resonances of groups of the same amino acids at different locations in a sequence. Since the TnI fragment used by Grand contained 3 Lys (98, 105, 107), one more than is present in the peptide used by Cachia *et al.* (1983) (Lys-105, Lys-107), it may be that this extra Lys (residue 98) accounts for the disagreement. It is important to note that the TnI sequence (104-115) is the minimum sequence for inhibitory activity of TnI, but it has not been determined to be the minimum sequence required for TnC binding. It is possible that residues (96-103) have a role in the TnI-TnC interaction.

The synthetic peptides of the TnI inhibitory region (104-115) used in this study bound to a TnC affinity column in the presence of Mg^{2+} or Ca^{2+}. Katayama and Nozaki (1982) demonstrated that a synthetic peptide of the TnI sequence (101-115) bound TnC in the presence of Mg^{2+} regardless of the concentration of Ca^{2+}. However, TnC did not bind to a TnI peptide affinity column in 100 mM EDTA (Chapter 4), but was bound to TnI peptide in solution when the peptide-protein interaction was monitored by circular dichroism (Chapters 6 and 7). This contradiction can be explained since a higher concentration of EDTA (100 mM) was required to elute the peptide from the TnC affinity column than required (1 mM) to remove metal ions from native TnC. Therefore, the higher ionic strength of the EDTA solution required for affinity chromatograph could have been involved in weakening the peptide-TnC interaction in the absence of cations. This suggests that the conformation of TnC in the absence of divalent cations is such that the putative

binding site for these peptides has an 'incorrect' spatial conformation so the interaction between the peptide(s) and TnC is very weak compared to that which occurs when Mg^{2+} or Ca^{2+} is present. It is well documented that TnC has a different conformation and the strength of the TnI-TnC (IC) interaction is much weaker in the absence of cation then when Mg^{2+} or Ca^{2+} is present (Johnson and Potter, 1978; Nagy and Gergely, 1979; Leavis *et al.*, 1978; Ingraham and Swenson, 1984; Cheung *et al.*, 1987).

It is interesting that after TnC was specifically removed from skeletal myofibrils in low ionic strength and in the presence of EDTA (Cox *et al.*, 1981; Zot and Potter, 1982), TnC could be rebound to the TnC-depleted myofibrils only when the Ca^{2+}-Mg^{2+} sites were filled, resulting in the restoration of the Ca^{2+}-induced activity of the myofibrils (Zot and Potter, 1982). This implies that there is an IC interaction that requires the specific TnC conformation that is induced in TnC when the Ca^{2+}-Mg^{2+} sites are filled. This IC interaction is probably the same as the Ca^{2+}-Mg^{2+} site-dependent interaction of TnC and the TnI inhibitory peptides seen in this study. Since previous studies (Robertson *et al.*, 1981) showed that the Ca^{2+}-Mg^{2+} sites are filled under normal physiological conditions, Zot and Potter (1982) suggested that the Ca^{2+}-Mg^{2+} sites have a structural role in stabilizing the Tn complex in a conformation ready for Ca^{2+} to bind to the Ca^{2+}-specific sites and contraction to occur. However, recently our laboratory demonstrated that Tn efficiently inhibits the acto-S1 ATPase activity only if the Ca^{2+}-Mg^{2+} sites are filled with a divalent cation (Van Eyk *et al.*, 1986). In the presence of EDTA, the inhibition induced by Tn was greatly reduced, indicating that the inhibitory region of TnI (104-115) had a weaker binding affinity for tropomyosin-actin as well as being less able to induce the conformational change in the thin filament. These results suggest that the Ca^{2+}-Mg^{2+} sites have not only a structural role in maintaining the integrity of Tn but that the conformation of Tn when these sites are filled, allows the inhibitory region of TnI to interact more efficiently with tropomyosin-actin. The present study shows that the synthetic TnI

inhibitory peptide (104-115) could bind to either TnC or tropomyosin-actin (Tables 8 and 9) in the presence of Mg^{2+}. This suggests that in the presence of Mg^{2+} the inhibitory region of TnI within the tropomyosin-troponin complex is restrained from interacting with TnC and therefore interacts with tropomyosin-actin.

The TnI inhibitory peptide (104-115) also displayed an increased binding affinity for TnC in the presence of Ca^{2+}, compared to Mg^{2+}, as seen from the increased KCl concentration required to elute the peptide from the affinity column (Table 9). This may simply reflect a different conformation at the Ca^{2+}-Mg^{2+} sites in the presence of Ca^{2+} and/or the binding of Ca^{2+} to the Ca^{2+}-specific sites altering the interaction of the peptide at the Ca^{2+}-Mg^{2+}-dependent binding sites on TnC (Wang and Gergely, 1986). It has been shown that there is an increase in the stability of the IC complex when the Ca^{2+}-specific sites are filled along with the Ca^{2+}-Mg^{2+} sites of TnC (Ingraham and Swenson, 1984; Cheung *et al.*, 1987).

As well, the glycine substituted analogs indicate that in the presence of Ca^{2+}, there is an accompanying shift toward a more specific interaction between the C-terminal amino acid residues of the TnI inhibitory peptide and TnC (Table 9). It is the C-terminal amino acids of the peptide which are also essential for tropomyosin-actin binding and the consequential inhibition of the ATPase activity (Table 8). This study indicates that the Ca^{2+}-dependent switch between muscle relaxation and contraction involves a switch of the inhibitory region of TnI between tropomyosin-actin and TnC. Although this site is of major importance, it is not the only Ca^{2+}-sensitive IC binding site (Grabarek *et al.*, 1981; Weeks and Perry, 1978) and thus may constitute only one aspect of the Ca^{2+} switch.

RECENT DEVELOPMENTS

One of the fundamental questions in biochemistry is the relationship between structure and function of active sites of proteins. By combining bioassays using Gly

substituted TnI analogs (work presented in this chapter) which determine the critical residues involved in the TnC interaction, with modern NMR structural methods it is possible to correlate structure and function of a specific region of a protein. Recently, the structure of the skeletal TnI peptide (104-115) bound to the Ca^{2+} saturated turkey skeletal TnC was determined using the NMR technique of transferred nuclear Overhouser effect (trNOE) (Fig.16, Campbell and Sykes, 1991). The trNOESY is an extension of NOE experiment to include exchanging systems such as ligand-protein complexes, and provides one of the best techniques for the determination of the structure of a bound ligand. Assuming fast exchange and a low protein concentration (5 to 10% relative to peptide concentration), the chemical shifts and linewidth are dominated by the population of free peptide, since the equilibrium concentration of free peptide is so much greater than the equilibrium concentration of bound peptide. Conversely, the crosspeak intensity in the trNOESY spectra is dominated by the bound population, for although the equilibrium concentration of bound peptide is low, the trNOE cross-peak intensity is dominated by the fast cross relaxation rates in the bound conformation, which is a function of the longer correlation times experienced in the bound as opposed to the free peptide. The long correlation times and faster cross relaxation rates produce strong negative NOE's which convey structural information about the bound peptide. The trNOE-derived structure of the skeletal TnI peptide bound to TnC is bent at the two proline residues which have ψ, ϕ values of approximately -70° and -170°. The bend around Pro 109 and 110 brings the residues on the hydrophobic face of the TnI peptide into close proximity. The ring protons of Phe 106 are close enough to the β-methyl protons of Val 114 to allow the observation of trNOE cross-peak between these two residues. Thus, the TnI peptide has a small hydrophobic pocket with the aromatic side chain of Phe 106 being somewhat buried by the side chains of Leu 111 and Val 114. The side chains of the basic residues extend outwards away from this hydrophobic pocket for interaction with TnC.

The work discussed in this chapter using the single Gly substitution analogs of TnI

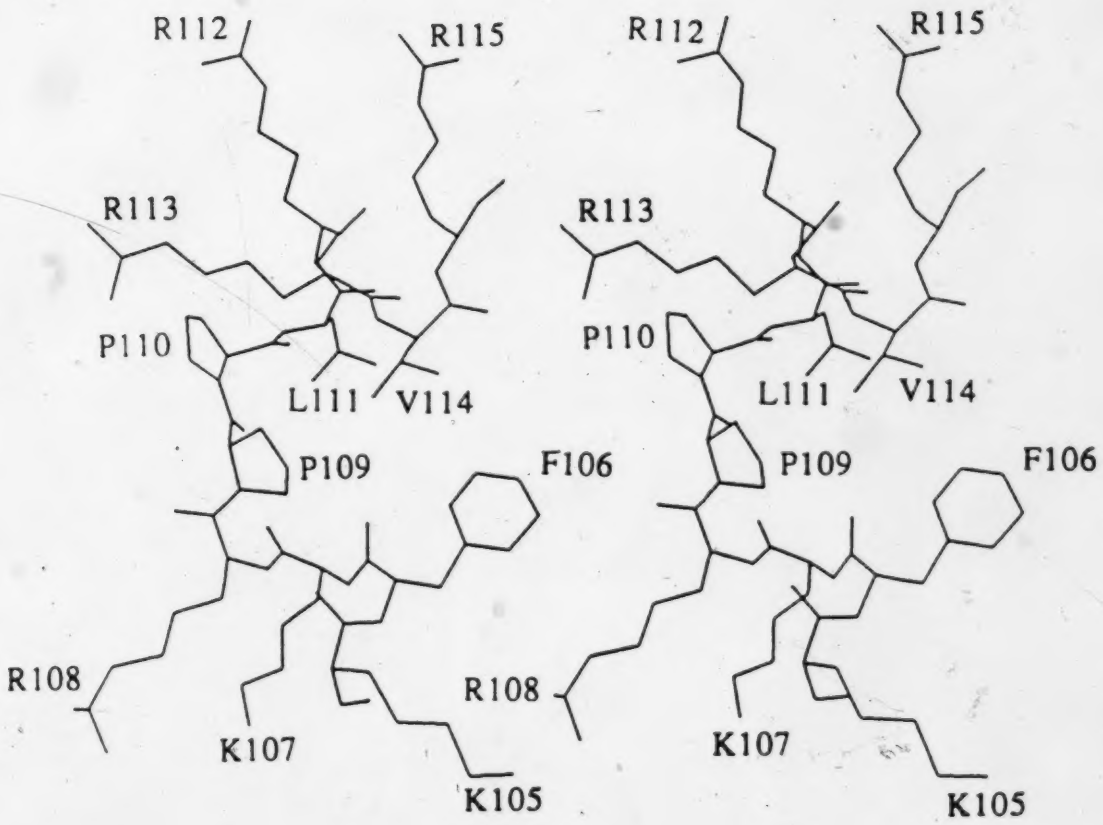

Figure 16. Stero-diagram of the TnI inhibitory peptide (104-115) bound to Ca^{2+} saturated TnC (Campbell and Sykes, 1991).

peptide (104-115) shows that the interaction between the peptide and Ca^{2+} saturated TnC involves contributions from both hydrophobic and electrostatic side chains. Identification of the residues which are the most critical for binding the peptide to TnC makes it possible to partially determine the positioning of the TnI peptide on TnC. The Gly substituted TnI

analogs which required the lowest concentration of KCl to be eluted for the Ca^{2+} saturated TnC HPAC column (Table 9) indicate the amino acid side chains which are important for the TnI peptide interaction with TnC in the presence of Ca. The most critical residues, in order of importance are Arg 115 > Leu 111 and Phe 106 > Val 114 and Arg 112. It is clear that the amino acid comprising the hydrophobic core of the TnI peptide (residues Phe 106, Leu 111 and Val 114) are extremely critical. Either the hydrophobic pocket is critical to stabilize the bent conformation of the TnI peptide so that other residues can better interact with TnC, and/or the residues of the hydrophobic pocket are directly interacting with other hydrophobic residues of TnC. It is of interest that Arg 112 and 115 are situated parallel to one another and in close proximity to Leu 111 and Val 114. This suggests that the critical points of contact between the TnI peptide and TnC are dominated by a relatively small area on the TnI peptide and not spread though out the peptide.

Modelling of the TnI peptide-TnC complex based on Gd relaxation studies (Campbell *et al.*, 1989, Campbell and Sykes, 1991) and cross-linking studies (Lesyzk *et al.*,1987) has the peptide situated in contact with the hydrophobic pocket located in the the C-terminal domain of TnC which is close to helix E. It is conceivable that the hydrophobic pocket of the TnI peptide would interact with the hydrophobic pocket of TnC, allowing ring stacking between the aromatic ring of Phe 106 of TnI peptide and the aromatic rings of any number of exposed Phe residues on TnC [Phe 105, 151 and 154 are exposed inside the hydrophobic pocket of the C-terminal domain of the avian crystal structure (Herzberg and James, 1985)]. As well, the side chains of the Leu 111 and Val 114 of the TnI peptide could interact with hydrophobic groups which line the pocket of TnC, and the basic side chains of the TnI peptide could interact electrostatically with a number of acidic groups located in helix E of TnC.

Comparison of the binding of the Gly TnI analogs to TnC HPAC column in Mg^{2+} (high affinity sites filled) and Ca^{2+} (high and low affinity sites filled) indicate the amino

acid residues that are critical for the switch between weak binding in the presence of Mg^{2+} and the strong peptide-TnC interaction in the presence of Ca^{2+}. Amino acid residues Val 114, Leu 111, Arg 115 and Pro 110 are the residues most effected by whether TnC has two or four divalent cations bound. These amino acid residues are located in the TnI peptide structure in such a way that they form a line through the middle of the peptide. This suggests that the transition between the Mg^{2+} and Ca^{2+} bound forms of the peptide involves movement around the center portion of the peptide affecting the position of these residues with respect to TnC . Movement around the center of the molecule would affect the extent to which the peptide is kinked at Pro 110. Therefore, the peptide could accommodate the structural change in the binding interface of TnC in the presence of Mg^{2+} as opposed to Ca^{2+}. Furthermore, substitution of Pro 110 with either Thr or Gly has a profound effect on the peptide-induced changes in the secondary structure and Ca^{2+} binding affinity of TnC (discussed in Chapter 7).

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CHAPTER 6

INHIBITION OF TnI-TnC INTERACTION AND CONTRACTION OF SKINNED MUSCLE FIBRES BY THE SYNTHETIC TnI PEPTIDE [104-115]

INTRODUCTION

Skinned muscle fibre studies were performed in order to investigate the effect of the synthetic TnI peptide 104-115 under more physiological conditions than acto-S1 ATPase assays. In skinned muscle assays, TnI peptide and mastoparan (Table 10) compete with TnI for its target proteins, in particular TnC. It was clearly shown that the TnI peptide can successfully compete with TnI for TnC in solution (thereby inhibiting the TnI-TnC (IC) interaction) by monitoring changes in the circular dichroism spectra (α-helical content) with various Ca^{2+} concentrations. Since both skeletal and cardiac muscle are regulated by the TnI-TnC interaction, we investigated whether contractile activation of the both types of skinned muscle fibres could be inhibited by TnC-binding peptides. All the skinned muscle fibre assays were preformed by Dr. J.C. Rüegg at the Univerisät Heidelberg, Heidelberg. This study was published in 1989 in Pflüger Arch: Eur. J. Physiol. 414, 430-436.

Table 10: Amino acid sequence of TnI peptide and mastoparan

TnI Peptide [104-115]	Ac-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-amide
Mastoparan	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-amide

RESULTS

CIRCULAR DICHROISM SPECTRA ANALYSIS OF THE TnC AND TnI PEPTIDE INTERACTION

Figure 17 shows the CD spectra values, measured at 222 nm, for TnC in the presence of the TnI inhibitory peptide (Table 10), and illustrates the change in ellipticity at this wavelength for varying concentrations of Ca^{2+}. Graphical summation of the spectra of TnC and the TnI peptide yields the theoretical values which would occur if complex formation did not occur or if complex formation occurred but did not involve any structural changes. A comparison of the observed Ca^{2+} titration curve at a 1:1 molar ratio of TnC to peptide, with the theoretical curve, shows that as a consequence of the increased negative ellipticity in the experimental relative to the theoretical curve, additional helix was induced as a result of complex formation.

The TnI peptide contains minimal helical structure at the concentrations of Ca^{2+} used in these experiments (data not shown and Chapter 7), even in the presence of the strong helix inducer, 50% TFE (trifluoroethanol) (Cachia *et al.*, 1986; Chapter 7), indicating that any increase in negative ellipticity upon complex formation must be the result of an increase in the number of helical residues within TnC. By utilizing the ellipticity values at the extreme ends of the Ca^{2+} concentrations used in this study, the number of helical residues in the minus (below 10^{-8} M) and plus (above 10^{-5} M) Ca^{2+} states could be determined for TnC and TnC-peptide complex (Table 11). Thus, the difference between the number of residues in a helical conformation that are induced in TnC by the binding of the peptide in the absence and presence of Ca^{2+} (7 residues (65 to 72) and 13 residues (93 to 106) were induced, respectively) can be attributed to the presence of the peptide and its influence on the secondary structure of TnC. It must be noted that the change in ellipticity in the presence versus the absence of Ca^{2+} may not be totally due to an increase in the

amount of α-helix but may also be due to a rearrangement or tightening of preexisting helices. Cachia *et al.* (1986) previously reported a slightly lower number of helical residues (3 to 4) induced into TnC by the peptide. However, it is clear that with respect to the amount of α-helix induced in TnC by Ca^{2+}, the binding of the TnI peptide involves a smaller change in the conformation of TnC.

As shown in Figure 17, the α-helix content of TnC as well as the TnI peptide-TnC complex is a function of Ca^{2+} concentration. The pCa^{2+} value which indicates the induction of half the Ca^{2+} dependent α-helices ($=pCa_{50}$) has been indicated by vertical bars through the curves. Note that there was a marked leftward shift in the Ca^{2+} titration curve of TnC after addition of a molar equivalent of TnI peptide. As listed in Table 11, there was a pCa_{50} shift of 0.13 units when the TnI peptide complexed with TnC (6.85 as compared with 6.98 for TnC and the peptide-TnC, respectively). The PCa_{50} values correspond to Ca^{2+} binding constants calculated from the CD data of $7.8 \times 10^6\ M^{-1}$ for TnC and $10 \times 10^6\ M^{-1}$ for the peptide-protein complex. The apparent Ca^{2+} binding constants, which were calculated from the CD measurements of TnC (and TnC-TnI) in Ca^{2+} titration experiments, were lower than those obtained from equilibrium dialysis by Potter and Gergely (1975). However, the ellipticity values and the number of helical residues for TnC and the TnI peptide-TnC complex were very similar to those previously measured by circular dichroism in the study of Cachia *et al.* (1986). It is clear from the circular dichroism spectra for a mixture of TnI-peptide and TnC shown in Figure 17 that complex formation induces, in the presence of Ca^{2+}, an increase in the number of helical residues in TnC as well as a minor pCa shift.

CIRCULAR DICHROISM SPECTRA ANALYSIS OF THE TnI-TnC AND TnI PEPTIDE (104-115) INTERACTION

Figure 17 also shows the Ca^{2+}-dependence of the CD spectra measured at 222 nm for the TnI-TnC complex and of the TnI-TnC plus TnI peptide mixture. As can be seen,

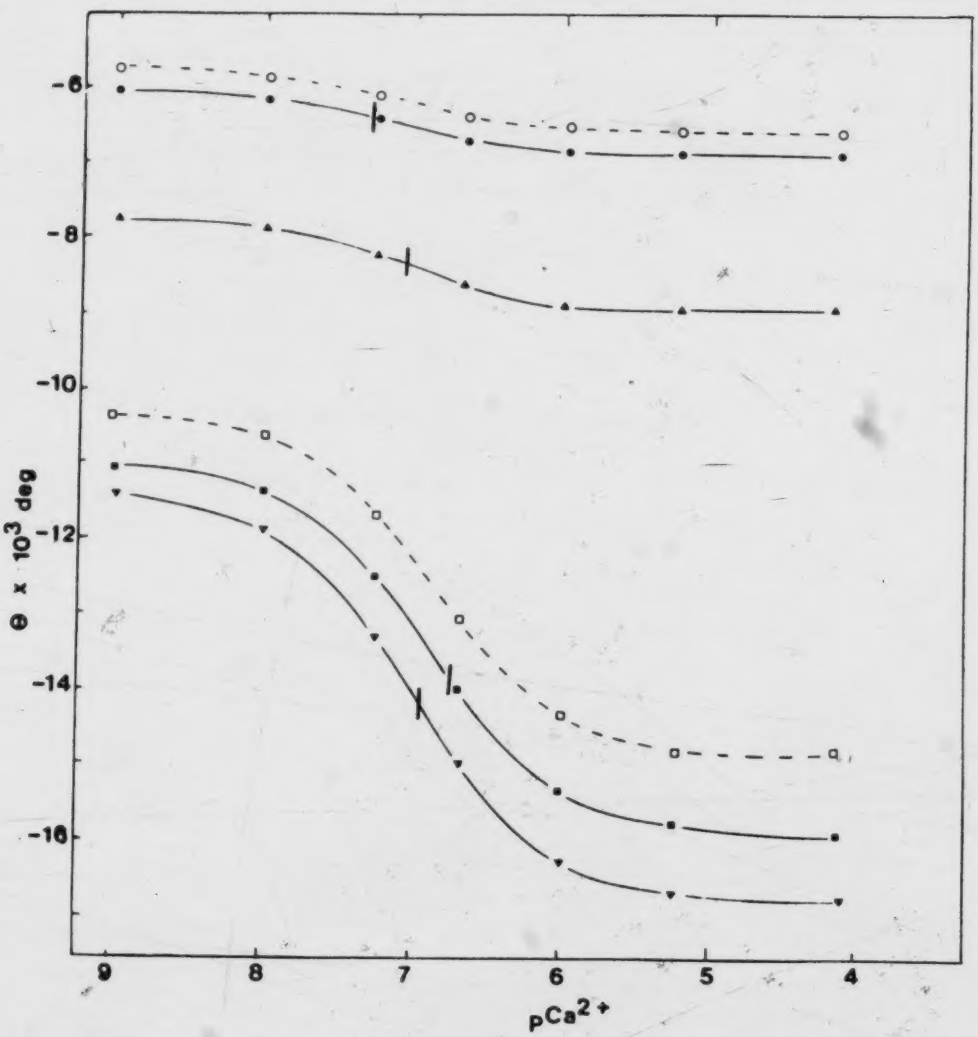

Figure 17. Effect of Ca^{2+} and TnI peptide on circular dichroism spectra at 222 nm of TnC and IC complex. Buffer contained 100 mM NaCl, 0.1 mM EGTA, 25 mM HEPES, pH 7.1 with increasing concentrations of Ca^{2+}. TnC observed (■), TnC plus 1 molar equivalent of TnI peptide observed (▼) and theoretical (□), IC observed (●), IC plus 1 molar equivalent of TnI peptide observed (▲) and theoretical (o). Bar through curves is the pCa^{2+} of the protein(s) which represent the concentration of Ca^{2+} required to induce half the Ca^{2+}-dependent α-helices.

Table 11. Structural changes induced by Ca^{2+} and TnI peptide in TnC and IC complex.

Protein	Presence or Absence of Metal Ion	$[\theta]_{222}$[a]	Number of Helical Residues[b]	Helical Residues Induced[c]	pC_{50}[f]	Apparent Ca^{2+} Binding Constant[d] (M^{-1})
TnC	$-Ca^{2+}$	-11100	65	28	6.85	7.8×10^6
	$+Ca^{2+}$	-15900	93			
TnC + peptide[e]	$-Ca^{2+}$	-11400	72	34	6.98	10×10^6
	+Ca2+	-16700	106			
IC	$-Ca^{2+}$	-6000	75	11	7.28	15.5×10^6
	$+Ca^{2+}$	-6900	86			
IC + peptide[e]	$-Ca^{2+}$	-7800	100	16	7.06	10.6×10^6
	$+Ca^{2+}$	-9000	116			

[a] Molar ellipticity values measured in 100 mM NaCl, 1 mM EGTA, 1 mM DTT, 25 mM HEPES, pH 7.11.
[b] Calculation of helical content as described in methods.
[c] Number of residues of α-helix induced by Ca^{2+} determined by comparing number of residues in protein in the absence compared to the presence of saturating Ca^{2+}.
[d] Apparent Ca^{2+} binding constant was determined by non-linear regression of CD data.
[e] TnI peptide to TnC ratio is 1:1.
[f] pCa_{50} required to induce half the helical change

the pCa_{50} value for the titration of the TnI-TnC complex is higher than that of TnC alone (7.28 versus 6.85), whereas it is intermediate for the mixture of the TnI-TnC complex and equimolar TnI-peptide (pCa_{50}=7.06, see Table 11). Thus, after addition of the TnI

peptide to the TnI-TnC mixture, there is a rightward shift of the titration curves by 0.22 pCa units towards the values of the TnI peptide-TnC complex (pCa_{50}=6.98) and away from the TnI-TnC value. This means that the Ca^{2+} binding constant of the TnI-TnC complex also decreases (from 15.5 x 10^6 M^{-1} to 10.6 x 10^6 M^{-1}) after addition of the peptide. Significantly, this value is very close to that of the TnC-peptide mixture (10 x 10^6 M^{-1}). These findings, in conjunction, are taken to mean that the TnI peptide introduced into a solution of pre-formed TnI-TnC complex competes with the corresponding region of TnI (residues 104-115) for a binding site on TnC, thereby forming the TnI peptide-TnC complex.

The influence of the peptide on TnI-TnC interaction can also be seen by the changes in the number of helical residues present in TnC. Ca^{2+} induced an increase in the number of helical residues in TnI-TnC from 75 to 86 residues (Table 11). In the presence of the TnI peptide, Ca^{2+} induced an additional increase in the number of residues in a helical conformation from 100 to 116. This implies that an additional 5 residues were induced into helices as a result of the TnI peptide's interfering with the TnI-TnC complex, which is approximately the same number of additional residues induced into helices seen previously in the peptide-TnC complex (6 residues). Thus the TnI peptide can compete successfully with TnI for TnC. It is interesting that there was no further increase in the observed ellipticity values at ratios of peptide to TnI-TnC of 2 and 10 molar equivalents, which suggests that the peptide has its maximum effect at approximately a 1:1 molar ratio with TnC.

In conclusion, both the increase in ellipticity and the subsequent decrease in Ca^{2+} affinity of TnC in the TnI peptide-TnI-TnC mixture (10.6 x 10^6 M^{-1}) with respect to the TnI-TnC complex (15.5 x 10^6 M^{-1}) indicate that the TnI peptide competes with TnI for TnC and successfully inhibits the TnI-TnC interaction. Next, we investigated the functional consequences of these peptide-induced alterations of TnI-TnC interactions in skinned fibres.

THE INTERACTION OF SKINNED SKELETAL MUSCLE FIBRES WITH TnI PEPTIDE (104-115)

The addition of the TnI peptide to skinned rabbit psoas fibres resulted in the inhibition of the isometric contractile responses elicited at 2.5 μM Ca^{2+} (Figure 18). The peptide-induced inhibition was reversible and dose-dependent and this dependence of the contractile force on the concentration of the TnI peptide is clearly illustrated in Figure 19.

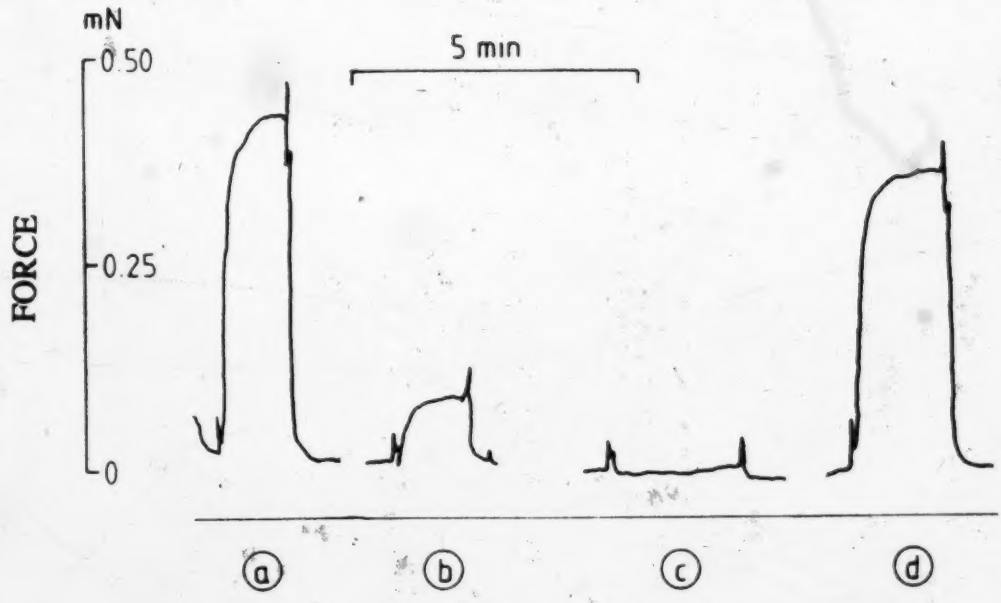

Figure 18. Inhibition of contraction of skinned psoas fibres by TnI peptide. Isometric contractile responses of a single skinned fibre were elicited by 2.5 μM Ca^{2+}. Relaxation was induced by immersion into relaxing solution (for composition of solutions see "Methods"). At b and c are the responses when inhibited by 50 μM and 100 μM of the peptide, respectively (the fibres were exposed to the peptide in relaxing solution for 15 min before eliciting the contraction with Ca^{2+}). A is the force development trace prior to addition of the peptide while d is the trace after the peptide has been washed from the fibres (assay performed by Dr. Rüegg).

The half maximal inhibition was achieved at an approximate TnI peptide concentration of 30 μM, whereas full inhibition of force development occurred at a concentration of 50 to 100 μM. In addition, the TnI peptide altered the Ca^{2+} force relationship of the skinned skeletal muscle fibres. This is demonstrated in Figure 20A which shows the effect of increasing Ca^{2+} concentration on the isometric force development of skinned rabbit psoas fibres in the absence and presence of the TnI-peptide. The peptide inhibited both the maximally Ca^{2+}-activated force as well as submaximal contractions. In Figure 20B, force values were normalized with respect to activation at high Ca^{2+} concentrations and plotted versus Ca^{2+} concentration. At a concentration of 50 μM, the peptide induced a rightward shift of the calcium-free relationship, showing that the apparent calcium sensitivity of the

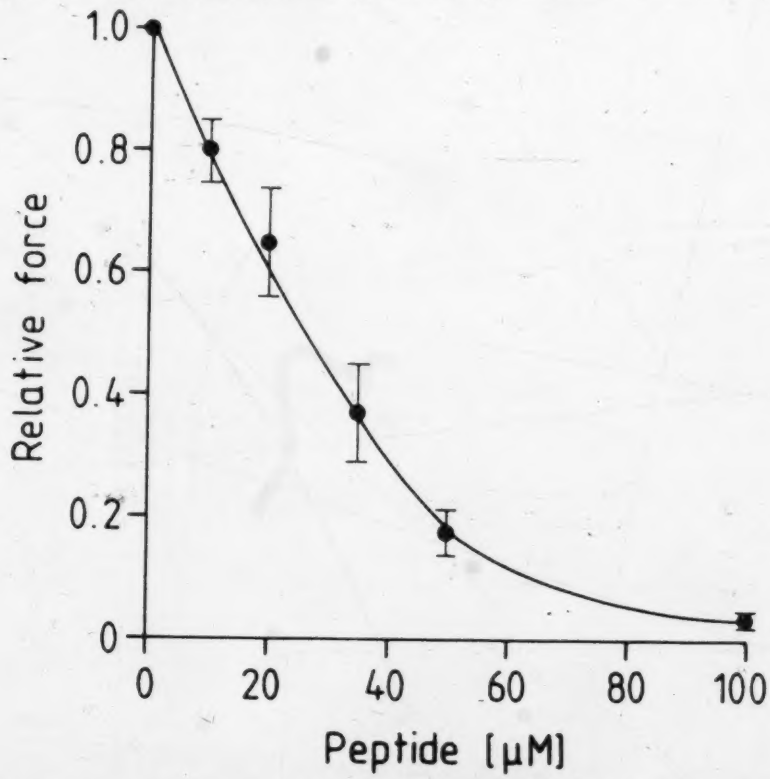

Figure 19. Dose dependence of TnI peptide inhibition in skinned psoas fibres. Plot of relative force elicited by 2.5 μM Ca^{2+} as a function of the concentration of the TnI peptide. Circles indicate relative force as a fraction of force obtained in the absence of the peptide (mean ± standard error (SE) of 3 to 6 experiments) (assay performed by Dr. Rüegg).

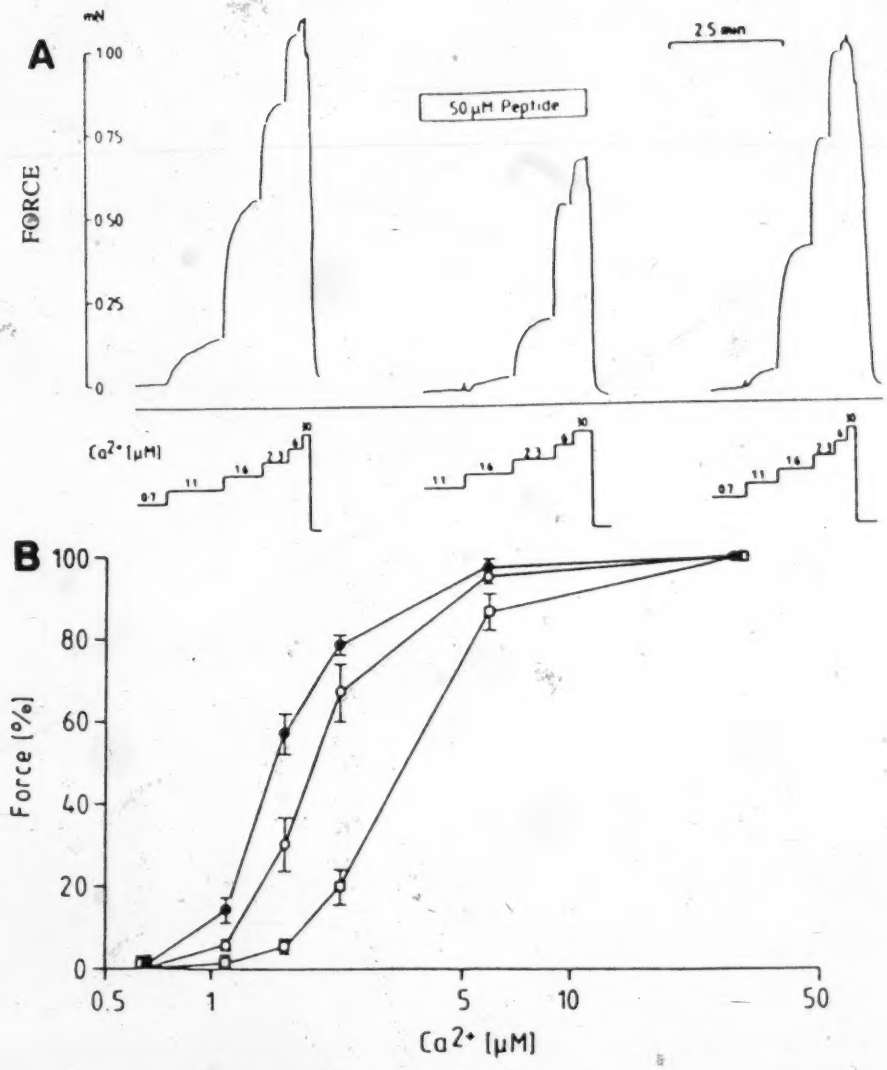

Figure 20. The TnI peptide decreases Ca^{2+} sensitivity in skinned psoas fibres. (A) Effect of cumulative increase in Ca^{2+} concentration on the isometric force development of a single skinned psoas fibre (diameter 50 µM) in the presence and the absence of 50 µM peptide and after the peptide has been washed out of the fibre. The concentration of Ca^{2+} at each addition is shown in the step graph shown along the x axis.. Note the depression in force induced by the peptide which can be restored after washing out. (B) Ca^{2+}-force relationship in the absence of the peptide (●) and in its presence (50 µM) following pre-incubation with the peptide (□) in relaxing solution and after washing out the peptide (o). Note that the peptide increases the Ca^{2+} concentration required for 50% activation reversibly. Force values were normalized with respect to the responses at 30 µM Ca^{2+} in the presence or absence of the peptide in order to demonstrate the change in "Ca^{2+} sensitivity" (assays performed by Dr. Rüegg).

contractile system has decreased. Thus, a greater Ca^{2+} concentration was required to produce a given intermediate force when the TnI peptide was present than when it was absent. This corresponds to a shift of the calcium-force relationship by approximately 0.3 to 0.4 pCa^{2+} units toward lower pCa^{2+} values when the peptide was present. By contrast, a peptide with a different amino acid sequence (Ac-G-L-G-A-K-G-A-G-V-G-amide) had no effect on the calcium responsiveness of skinned fibers (data not shown).

THE INTERACTION OF CARDIAC MUSCLE FIBRES WITH THE TnI PEPTIDE [104-115]

Skinned muscle fibres from porcine right ventricle were also utilized to determine the effect of the TnI peptide on force development. The TnI peptide reversibly inhibited the tension development in skinned cardiac muscle. The dependence of the contractile force on the concentration of the TnI peptide was similar to that seen in skeletal muscle (Figure 21). However, at maximal Ca^{2+} activation (30 μM), the TnI peptide was not as efficient at inhibiting force generation in cardiac as opposed to skeletal muscle fibres (Table 12). There was a reduction in the tension development of only 26% in cardiac muscle, compared inhibiting force generation in cardiac as opposed to skeletal muscle fibres (Table 12). There was a reduction in the tension development of only 26% in cardiac muscle, compared to 38% in skeletal muscle at maximal Ca^{2+} activation.

As with skeletal muscle fibres the relationship between the Ca^{2+} concentration and force development was altered when the TnI peptide was present (Figure 22). Higher concentrations of Ca^{2+} were required to elicit the same amount of force in the presence of the peptide. This corresponded to a small but significant decrease in the pCa^{2+} value of approximately 0.15 pCa^{2+} units. The magnitude of the shift in Ca^{2+} sensitivity which is due to the interaction of the TnI peptide in cardiac muscle was approximately half that seen in skeletal muscle.

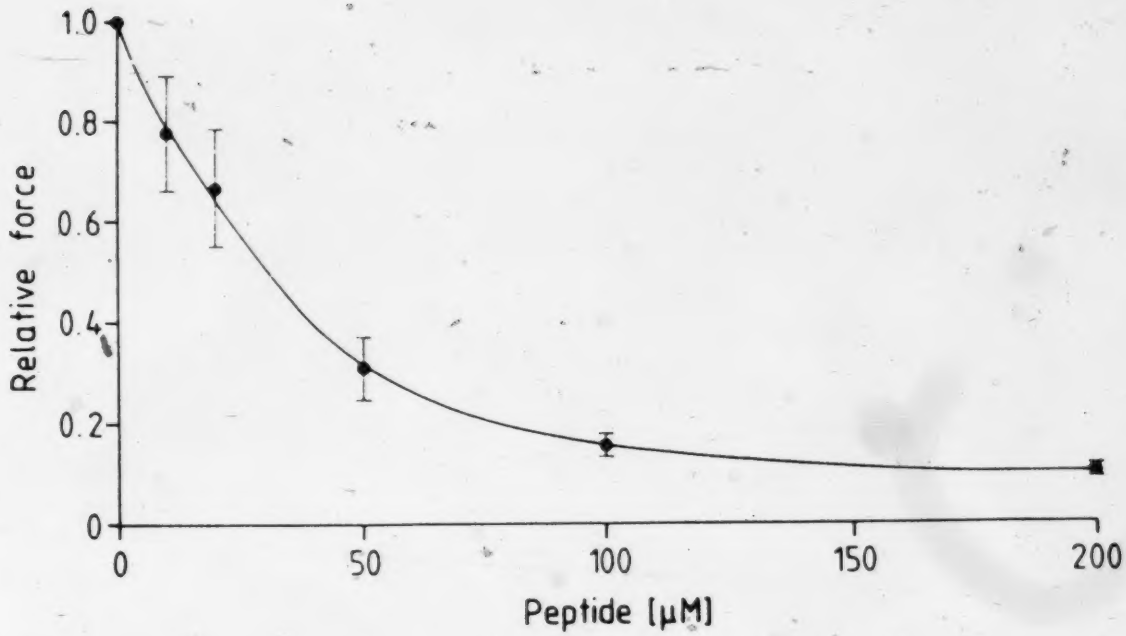

Figure 21. Dose dependence of TnI peptide inhibition in skinned pig cardiac fibres. Plot of relative force elicited by 2.5 μM Ca^{2+} as a function of the concentration of the TnI peptide. Solid circles indicate relative force as a fraction of force obtained in the absence of the peptide (mean ±SE of 3 to 6 experiments) (assay performed by Dr. Rüegg).

TABLE 12: Effect of TnI peptide 104-115 and mastoparan on the Ca^{2+}-activated force in skinned muscle fibres

		Inhibition (%)[a]	
Inhibitor	Ca^{2+} (μM)	Rabbit psoas	Pig cardiac (ventricle)
TnI (104-115)	30.0	37.1 ± 3.4 (5)	26.1 ± 37 (6)
TnI (104-115)	2.3	82.6 ± 3.6 (6)	69.3 ± 6.4 (4)
Mastoparan	1.6	43 ± 6.4 (5)	4.9 ± 4.9 (6)

[a] Decrease of force after addition of the peptide (% ± SE).

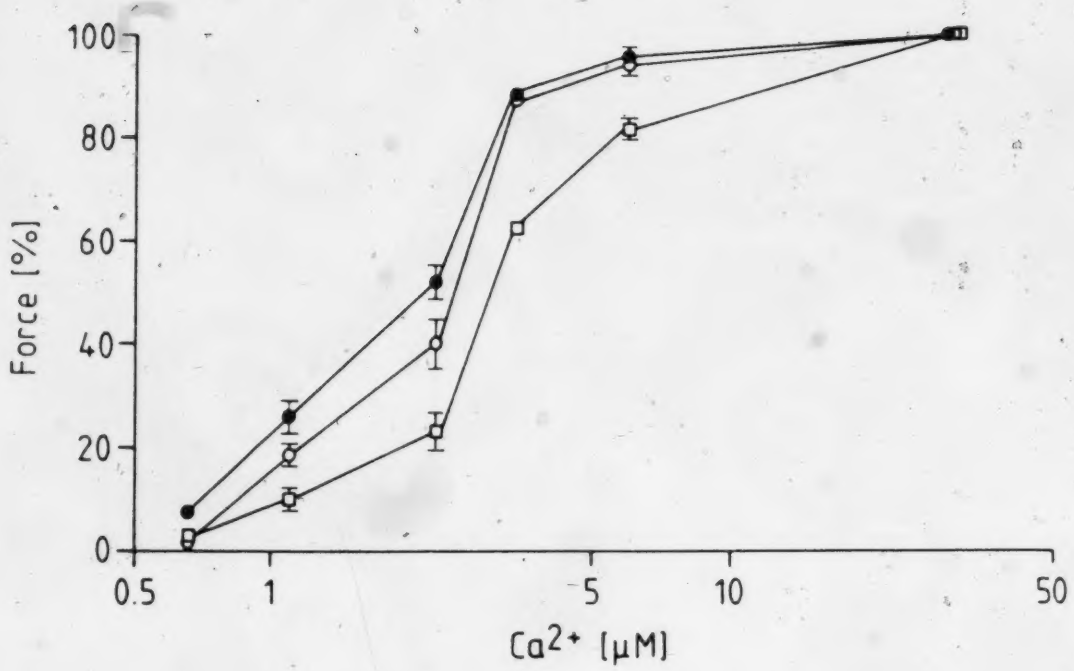

Figure 22. Ca^{2+}-force relationship of skinned pig cardiac fibres before addition of the TnI peptide (●) and in its presence (50 µM, □) (following pre-incubation with the peptide in relaxing solution), and after washing out the peptide (o). Note that the peptide increases the Ca^{2+} concentration required for 50% activation (force values normalized with respect to the responses at 30 µM Ca^{2+} in the presence or absence of the peptide in order to demonstrate the change in "Ca^{2+} sensitivity")(assays performed by Dr. Rüegg).

THE INTERACTION OF SKELETAL AND CARDIAC SKINNED MUSCLE FIBRES WITH MASTOPARAN

The mastoparan peptide was capable of partially inhibiting the contractile response in both skeletal (Figure 23) and cardiac muscle (Table 12). The magnitude of the inhibition induced by mastoparan in these muscles was substantially less than that induced by the TnI peptide. Therefore, mastoparan was less effective at inhibiting the TnI - TnC interaction (43% in skeletal muscle and 4.9% in cardiac muscle compared to 82.6% and 69.3% respectively for the TnI peptide, Table 12).

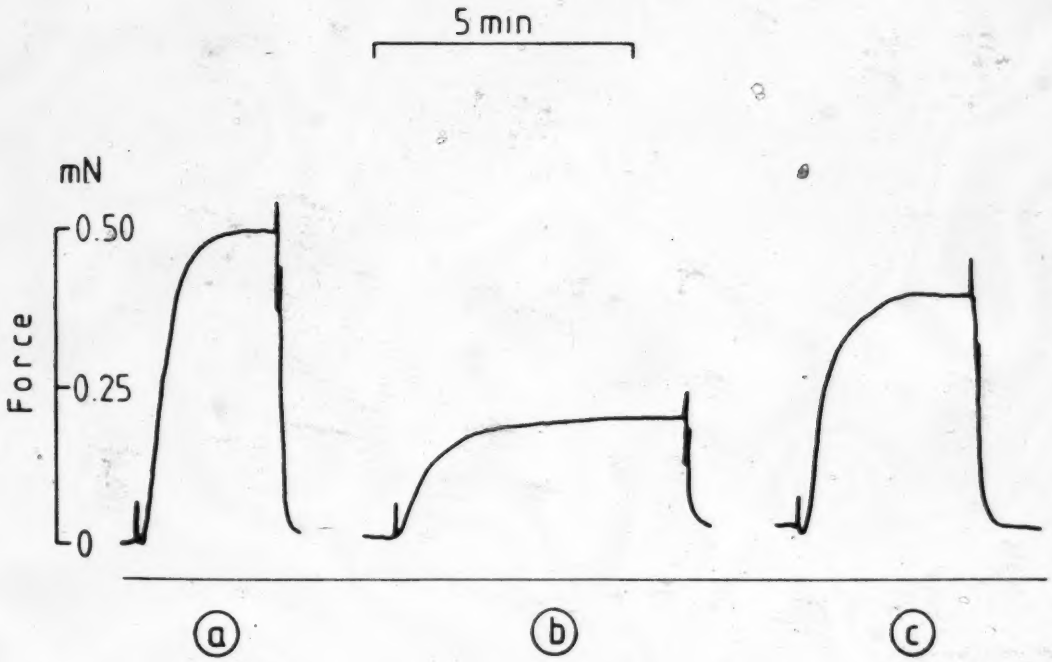

Figure 23. Inhibition of contraction of skinned psoas fibres by mastoparan. Contractile responses were elicited by 2.5 μM Ca^{2+}. At b the response was partly inhibited by 100 μM mastoparan (note that the fibre was also exposed to this peptide 15 min before eliciting contraction with Ca^{2+}). At a and c is the amount of force developed before addition of the peptide and after the peptide was washed from the fibre, respectively (assays preformed by Dr. Rüegg).

DISCUSSION

The circular dichroism spectra for a mixture of TnI peptide and TnC clearly show that complex formation induces an increase in the number of α-helical residues in TnC as well as a minor increase in Ca^{2+} affinity. The most intriguing CD result was the ability of the TnI peptide to alter the TnI-TnC interaction. The TnI peptide in the presence of the TnI-TnC complex caused an increase in the number of α-helical residues induced in TnC and a decrease in its apparent Ca^{2+} binding constant from that observed with TnI-TnC, toward

the values obtained for the TnI peptide-TnC complex. This data leads to the important conclusion that the TnI peptide inhibits the interaction between TnC and TnI.

Skinned muscles were investigated to determine the possible effect the inhibition of the TnI-TnC interaction by the TnI peptide would have on the Ca^{2+} dependence of force development. Since cardiac and skeletal muscle are regulated by the Tn system, both types of muscle were studied to compare the effect of the peptides. The TnI peptide inhibited the skeletal muscle fibre in a dose-dependent and reversible manner. As well there was a decrease in the pCa_{50} values required for 50% force development and a corresponding drop in the Ca^{2+} responsiveness when compared to the muscle fibre in the absence of the peptide. These results can most easily be explained in conjunction with the results obtained from the CD data, namely that the TnI peptide blocks the TnI-TnC interaction. Though CD measurements monitored primarily calcium-induced structural changes associated with the high affinity sites of TnC (Hincke *et al.*, 1978; Johnson and Potter, 1978; Nagy and Gergely, 1979), 20 to 38% of the structural change is attributed to Ca^{2+} binding at the Ca^{2+}-specific sites. As well, the TnC interaction with TnI increases calcium affinity of the Ca^{2+}-specific sites (Zot *et al.*, 1983). In Chapter 7, it is clearly shown that the skeletal TnI peptide 104-115 specifically increases the Ca^{2+} affinity of the sites I and II (Van Eyk *et al.*, 1991). However, since the TnI peptide can also bind to actin-TM and inhibits the acto-S1 ATPase activity (Talbot and Hodges, 1979, 1981), the peptide could conceivably be affecting the force development through this interaction. Which interaction plays the dominant role in the TnI peptide interaction in the skinned skeletal muscle depends on the relative binding constants of the peptide to TnC and to actin-TM. The addition of the TnI peptide produced a similar response in cardiac muscle fibres as it did in the skeletal system. However, force development was markedly less inhibited in cardiac than in skeletal muscle, as was the magnitude of the pCa^{2+} shift.

The effect of the TnI-peptide could be mimicked by mastoparan, a tetradecapeptide from the Vespid wasp venom which binds TnC (Malencik and Anderson, 1983). However, this peptide possesses many characteristics that differ from the TnI peptide. It forms a basic amphiphatic helix upon binding to TnC and induces more helical formation within TnC than that induced by the TnI peptide (Cachia *et al.* , 1986). TnC binds to mastoparan slightly better than does the TnI peptide and affects the Ca^{2+} binding to a greater degree (Van Eyk and Hodges, 1987). It is significant that NMR results suggest that both peptides bind at the same region, helix III of TnC (Cachia, Gariépy and Hodges, unpublished results, see Discussion in Chapter 4). Like the TnI peptide, mastoparan was also capable of partial inhibition of the contractile response in skinned skeletal and cardiac muscle fibres. However, the extent of inhibition of tension development by mastoparan was much less than that induced by the addition of the TnI peptide in either muscle type. This is not surprising since mastoparan is much less effective at inhibiting the skeletal acto-S1 ATPase activity when compared to the TnI peptide (Cachia *et al.*, 1986). Future work will have to show whether the contractile inhibition produced by TnI (104-115) and the mastoparan peptide will be better accounted for by an inhibition of the TnI-TnC interaction or by a more direct interaction with TM-actin.

RECENT DEVELOPMENTS

Recent work was done in collaboration with Dr C. Rüegg, Heidelberg Universität, Heidelberg, Germany, to determine whether the effect of the skeletal TnI peptide (104-115) on Ca^{2+} binding to Tnc in the skinned muscle fibres. A Danz (5-dimethylaminoaphthalene-1-sulfonyl aziridine) fluorescence group was attached to Met 25 of skeletal TnC and incorporated into TnC-depleted skinned muscle psoas muscle fibres (Zot and Potter, 1986). The force development of the muscle fibres and fluorescence of the TnC in response to two Ca^{2+} concentrations, 3.2×10^{-6} M (pCa 5.5) and 3.2×10^{-5} M (pCa 4.5), the latter

producing maximal activation, were determined in the absence and presence of the skeletal TnI peptide. The change in fluorescence of the Danz label monitors the Ca^{2+} binding at the regulatory low affinity sites of TnC (sites I and II). At submaximal Ca^{2+} concentrations

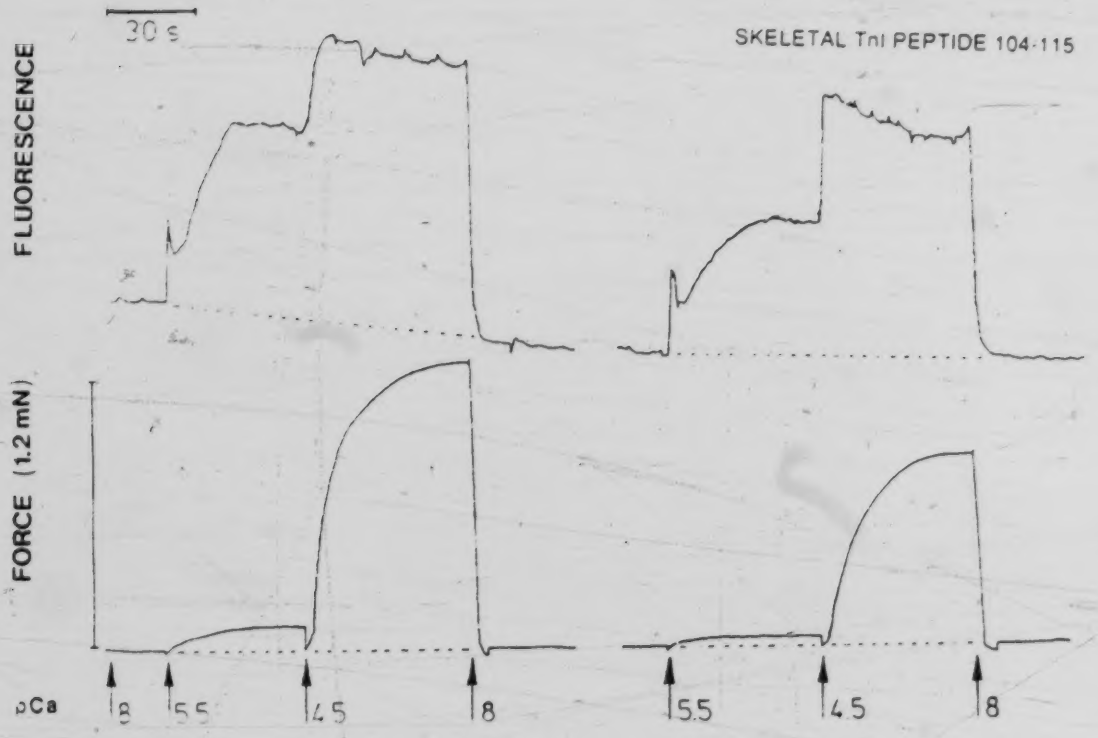

Figure 24. Fluorescence and force development in the absence and presence of TnI peptide (104-115) in psoas skinned muscle fibres. Danz labelled TnC was incorporated into TnC-depleted skinned muscle fibres according to Zot *et al.*, 1986. The Danz binds to Met 25 of TnC which is in the vicinity of Ca^{2+} binding site I and undergoes a Ca^{2+} induced fluorescence enhancement which is similar to Ca^{2+}-sensitivity displayed for Ca^{2+} binding at the low affinity sites (Johnson *et al.*, 1978). The fluorescence change of the incorporated Danz labelled TnC indicates that the presence of the TnI peptide in the skinned muscle fibres affects the Ca^{2+} binding to the low affinity sites (assays performed by Dr. Rüegg).

(pCa 5.5), the peptide inhibits the fluorescence of the Danz-TnC as well as force development in the skinned muscle fibre. The quenching of the fluorescence by 20-28% indicates that the peptide affects Ca^{2+} binding at the low affinity Ca^{2+} binding sites of TnC (Fig 24). At maximal Ca^{2+} concentrations (pCa 4.5), the TnI peptide inhibits force development but has minimal effect on the florescence of the Danz-TnC. One would not expect to detect a change in the Danz label at maximum Ca^{2+} concentrations since the low affinity sites are filled and the florescence of the Danz-labelled TnC should be at its maximum.

The work presented in this chapter was the first published paper concerning the use of synthetic peptides as probes in skinned psoas or cardiac muscle fibres (Rüegg *et al.*, 1989a). Many studies using synthetic peptides in skinned muscle fibres have been reported as preliminary findings at meetings (abstracts) or as short communications: a synthetic S1 peptide (residues 703-708) was reported by Suzuki et al. (1987) as a putative actin binding site on myosin, which inhibited contraction of skinned psoas muscle fibres (Chase and Kushmerick, 1989). A larger S1 peptide (residues 701-728) which bind tightly to actin were found to inhibit contractile force of skinned fibres as well as actomyosin ATPase activity (Rüegg *et al.*, 1989 b). A recent paper showed that synthetic peptides corresponding to sequences flanking the above region of S1 had either no effect or were increasing the Ca^{2+} responsiveness, thereby shifting the relationship between Ca^{2+} and force toward lower Ca^{2+}concentrations (Keane *et al.*, 1990). In vertebrate smooth muscle, where contractile activation depends on Ca^{2+} binding to CaM (which then interacts with myosin light chain kinase (MLCK)), a synthetic peptide containing the CaM recognition sites of MLCK (residues 493-512) inhibited force development in skinned smooth muscle fibre (Rüegg *et al.*, 1989c).

Peptide competition in skinned muscle fibres is a powerful tool that can be used to inhibit protein-protein interaction under more physiological conditions than in S1 or HMM

ATPase assays. However, the peptides are in competition with the native protein present in the muscle fibre, which leads to a higher degree of complexity than seen in the ATPase assays. Therefore, interpretation of the results obtained from skinned muscle fibre experiments is more difficult to understand and detailed knowledge at the molecular level of how the synthetic peptide interacts with its target molecules must be previously known.

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CHAPTER 7

A COMPARATIVE STUDY OF THE INTERACTIONS OF SYNTHETIC PEPTIDES OF THE SKELETAL AND CARDIAC TnI INHIBITORY REGION WITH SKELETAL AND CARDIAC TnC

INTRODUCTION

Some of the physiological differences in the action of skeletal and cardiac muscle may be explained in part by differences in the amino acid sequences of skeletal and cardiac TnI and TnC, especially at the binding interfaces between these highly conserved proteins. The differences in TnI and TnC could alter the IC interaction and hence affect muscle regulation. The skeletal and cardiac TnI inhibitory regions have identical amino acid sequences except at position 110 which contains Pro in the skeletal sequence and Thr in the cardiac sequence. In addition, skeletal and cardiac TnC have 65% amino acid homology with the majority of substitutions occurring in the first 40 residues. In fact, cardiac TnC has 8 amino acid differences located in Ca^{2+} binding site I, which makes this binding site nonfunctional. Hence, skeletal TnC binds 4 Ca^{2+} ions, while cardiac TnC binds only three Ca^{2+} ions. The high affinity sites III and IV are located in the carboxy-terminal half of TnC and bind Ca^{2+}, with an association constant of 2×10^7 and 3×10^8 M^{-1} for skeletal TnC and cardiac TnC, respectively (Potter and Gergely 1975; Johnson and Potter, 1978; Holroyde et al., 1980). Sites I and II in the amino-terminal half of the molecule bind Ca^{2+} with an association constant of 3×10^5 M^{-1} for skeletal TnC and 2×10^6 M^{-1} for site II of cardiac TnC.

Skeletal TnI is a more effective inhibitor than cardiac TnI in both cardiac and skeletal ATPase assays and skeletal TnC is more effective than cardiac in releasing the

inhibition induced by either cardiac or skeletal TnI (Talbot and Hodges, 1981a; Hincke et al., 1977). As well, the skeletal TnI peptide 104-115 is a better inhibitor of the skeletal or cardiac acto-myosin ATPase activity than the cardiac TnI peptide 137-148 (Talbot and Hodges, 1981a). The skeletal and cardiac TnI inhibitory peptides equally inhibit force development in skeletal and cardiac skinned muscle fibres, but the cardiac peptide is less efficient, requiring a 2 to 3-fold higher concentration to reach maximum inhibition (Rüegg et al., 1989 and 1991). In saturating Ca^{2+}, sIp dissociation constants for TnC range from 5×10^{-5} M to 1×10^{-6} M (Cachia et al., 1983a; Campbell and Sykes, 1991; Malencik and Anderson, 1984). Previous work shows that the binding of skeletal TnI inhibitory peptide 104-115 alters the secondary structure of skeletal TnC by increasing the α-helical content and producing a small pCa^{2+} shift (Rüegg et al., 1989). In this chapter (Biochemistry, in press), the effect of the skeletal and cardiac TnI peptides and a single Gly-substituted analog at position 110 (Gly 110)(Table 13) on Ca^{2+}affinity of skeletal and cardiac TnC are investigated by circular dichroism and Tyr fluorescence.

RESULTS

SECONDARY STRUCTURE OF THE TnI PEPTIDES IN SOLUTION

The CD spectra (190-250 nm) of the three TnI peptides, in the absence and presence of a 50% solution of the helix-inducing solvent trifluoroethanol (TFE), were carried out in order to determine whether the peptides possess secondary structure in benign solution, and whether secondary structure could be induced (Fig. 25). In the absence of 50% TFE, the three peptides produced CD spectra that were typical of random coils. In the presence of 50% TFE, the CD spectra of the peptides showed a minor increase in negative ellipticity at 222 nm of approximately -2000° A completely α-helical peptide of 12 residues would have a negative ellipticity of approximately -27,000°. In the absence and presence of TFE, it is interesting that the CD spectra of the three peptides

Table 13. Amino acid sequence of TnI inhibitory peptides

Peptide Name[a]	Abbreviated Name	Sequence[b]
Skeletal TnI Ac(104-115) amide [cardiac TnI Pro 143 Ac(137-148) amide]	sIp	Ac G-K-F-K-R-P-P-L-R-R-V-R-amide *
Cardiac TnI Ac(137-148) amide [skeletal TnI Thr 110 Ac(104-115) amide]	cIp	Ac-G-K-F-K-R-P-T-L-R-R-V-R-amide *
Skeletal TnI Gly 110 Ac(104-115) amide [cardiac TnI Gly 145 Ac(139-148) amide]	Gly 110	Ac-G-K-F-K-R-P-G-L-R-R-V-R-amide *

[a,b] Since the native sequences of skeletal and cardiac TnI in this region differ at only one position as denoted by the asterisk, *, the nomenclature for the peptides can be given in terms of the skeletal sequence or cardiac sequence. The alternative nomenclature is given in the brackets.

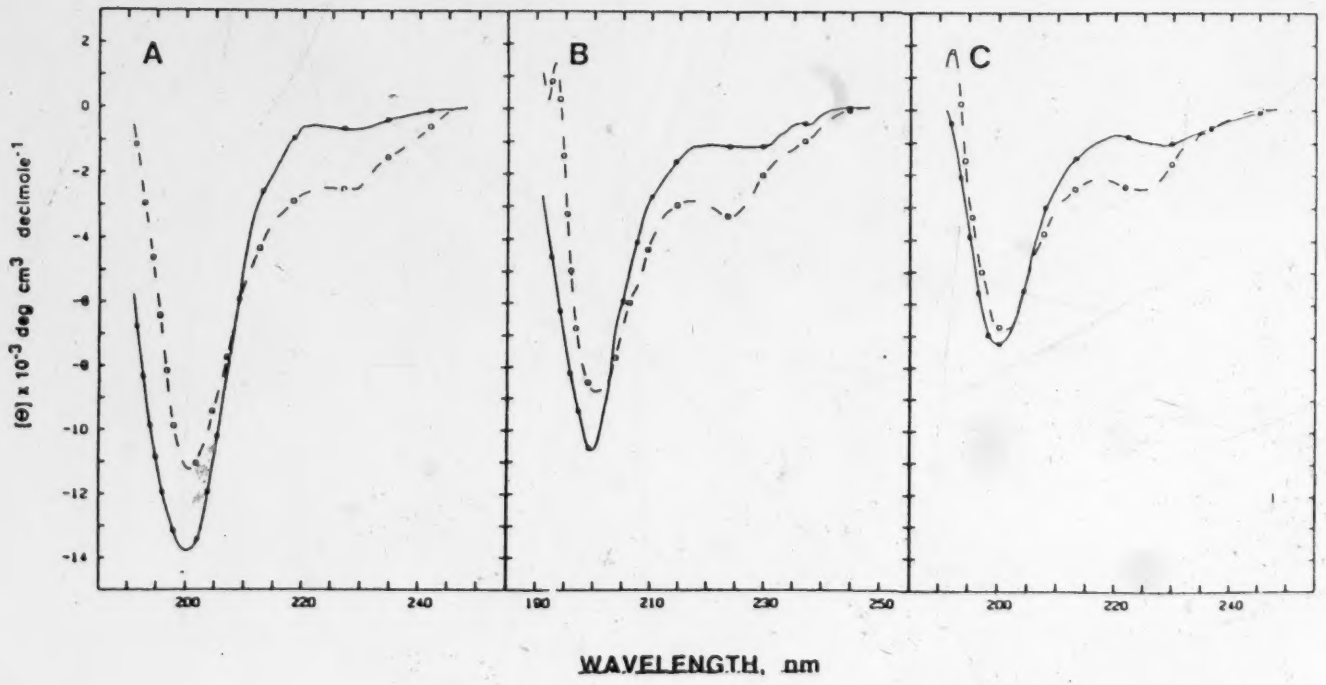

Figure 25. Circular dichroism spectra of TnI peptides in the absence and presence of 50% TFE. Spectra in the absence (-■-) and presence (-□-) of 50% TFE of sIp (panel A), cIp (panel B) and Gly 110 (panel C). Assay buffer 20 mM Mops, 50 mM KCl, 0.1 mM EGTA pH 7.2. Concentration of peptide were 1mM to which TFE was added to 50% v/v.

cannot be superimposed on one another, indicating they do not have the same conformation in solution. In addition, the ellipticity ratio (200/222 nm) in the presence of TFE varied for the 3 peptides: sIp, cIp and Gly 110 with values of 4.3, 2.8 and 2.8, respectively.

THE EFFECT OF THE TnI PEPTIDES ON THE SECONDARY STRUCTURE OF SKELETAL AND CARDIAC TnC

In the absence of Ca^{2+}, skeletal TnC has fewer helical residues (68) than cardiac TnC (74), while both have approximately the same number of helical residues in the presence of Ca^{2+} (108 vs 106 residues, respectively). Hence, Ca^{2+} binding to skeletal TnC induces an additional 40 helical residues compared with an additional 32 residues in cardiac TnC. This difference suggests that in the absence of Ca^{2+} cardiac TnC contains more structure than skeletal TnC, and undergoes a smaller Ca^{2+}-induced structural change. When Ca^{2+} binds to skeletal TnC, some of the α-helical residues that are induced are probably equivalent to helical residues which are already pre-formed in cardiac TnC in the absence of Ca^{2+}.

Since the three TnI peptides did not contain significant secondary structure, even in the presence of a helix-inducing solvent, one can assume that the interaction of the peptides with TnC does not induce helix in any of the peptides. Therefore, any change in ellipticity may be due to a change in the number of helical residues in TnC. As stated previously, the change in ellipticity in the presence versus the absence of Ca^{2+} may not be totally due to an increase in the α-helical content of TnC but may also be due to a rearrangement or tightening of preexisting helices. In this chapter the change in ellipiticity upon binding of the TnI peptide to TnC is analyzed as a change in the number of helical residues in TnC. This was done inorder to quantitate the conformational change that occurs upon interaction of the peptide and protein. The number of additional helical residues induced by the

Table 14. Number of additional α-helical residues induced in skeletal and cardiac TnC by the TnI peptides and Ca^{2+}

Presence or Absence of Metal Ion	Peptide[1] Added	ΔHR[2] sTnC	ΔHR[2] cTnC
$-Ca^{2+}$	sIp	+15	-4
$+Ca^{2+}$		+15	-3
$-Ca^{2+}$	cIp	+3	+5
$+Ca^{2+}$		+2	+3
$-Ca^{2+}$	Gly 110	0	+7
$+Ca^{2+}$		0	+1

Presence or Absence of Peptide	Metal Ion Added	ΔHR[2] sTnC	ΔHR[2] cTnC
-sIp	Ca^{2+}	40	32
+sIp		40	33
-cIp	Ca^{2+}	41	33
+cIp		40	30
-Gly 110	Ca^{2+}	39	32
+Gly 110		39	27

[1] A 1:1 mole ratio of TnI peptide was added to TnC.

[2] ΔHR is the difference in the number of helical residues induced by addition of peptide (upper section) or Ca^{2+} (lower section) in either skeletal or cardiac troponin C, sTnC or cTnC, respectively. Any change in the number of additional helical residues (ΔHR) induced greater than 2 residues is significant (see Method section).

peptides and Ca^{2+} in both skeletal and cardiac TnC is listed in Table 14. If the number of additional helical residues induced by the binding of Ca^{2+} is identical, whether the peptide is absent or present, then any additional helix induced when the peptide binds is unique and different from the helix induced when Ca^{2+}binds. On the other hand, if the number of additional helical residues induced by the binding of Ca^{2+} is different in the absence and presence of the peptide, then some of the peptide-induced helix include some residues that become helical when Ca^{2+} binds.

Skeletal TnI peptide (sIp) had the largest effect on the helical content in skeletal TnC. Binding of sIp resulted in the formation of an additional 15 helical residues in skeletal TnC in both the absence and presence of Ca^{2+} (Table 14). The 15 helical residues induced by sIp binding are in addition or, over and above, that structure induced by Ca^{2+} (40 helical residues were induced by Ca^{2+} in the absence and presence of peptide, Table 14). In other words, sIp causes unique structural changes in TnC. The binding of cardiac TnI peptide (cIp) to skeletal TnC had no effect on the number of helical residues induced upon Ca^{2+} binding (41 and 40 residues were induced in the absence and presence of cIp). and only a marginal (if any) effect on the helical content of TnC (3 and 2 residues in the absence and presence of Ca^{2+}, respectively). As well, Gly 110 does not alter the helical content of skeletal TnC (Table 14). This implies that cIp or Gly 110 may not bind TnC or that the interaction does not cause any significant change in the helical content of TnC. Both peptides, cIp and Gly 110, bind almost as tightly as sIp to a skeletal TnC affinity column in the presence of Mg^{2+} or Ca^{2+} (Van Eyk and Hodges, 1988, data not shown) and as shown in a later section the peptides alter the Ca^{2+} affinity of TnC. Therefore, cIp and Gly 110 peptides are able to bind TnC but are unable to significantly alter the secondary structure of skeletal TnC.

The effects of the three TnI peptides on the helical content of cardiac TnC are different from those observed with skeletal TnC. For example, Gly 110 which caused no change in the helical content of skeletal TnC induced some α-helical structure in cardiac

TnC in the absence of Ca^{2+}. The difference of 6 additional helical residues induced by Gly 110 in the absence as opposed to the presence of Ca^{2+} (7 residues minus 1 residue, Table 14), is similar to the difference of 5 helical residues induced when Ca^{2+} binds in the absence as opposed to the presence of Gly 110 (32 minus 27 residues). This suggests that most of the structure formed when Gly 110 binds is the same as the structure formed when Ca^{2+} binds. Cardiac TnI peptide induced a small change in the number of helical residues in the absence and presence of Ca^{2+} (5 and 3 residues were induced in the absence and presence of Ca^{2+}, respectively), while Ca^{2+} binding induced 33 helical residues compared to 30 in the absence and presence of the peptide, respectively. The difference in the number of helical residues induced by cIp is similar to the difference in the number of helical residues induced by Ca^{2+}. This indicates that part of the structure formed when cIp binds is the same structure formed when Ca^{2+} binds. The effect of cIp is minor (3-5 residues) compared to the effect of Ca^{2+} binding (30-33 residues). In some cases the change in secondary structure of TnC upon interaction of a peptide is small but there is good correlation between the difference in the number of residues induced by the peptide binding and that induced by Ca^{2+}. It is surprising that, skeletal TnI peptide which caused a large increase in helical structure in skeletal TnC (+15 residues) actually decreased the α-helical content of cardiac TnC (-4 and -3 residues in the absence and presence of Ca^{2+}). Since sIp does not alter the number of helical residues induced when Ca^{2+} binds, the majority of the structural changes induced by binding of sIp are not residues which are involved in the Ca^{2+}-dependent helix. The majority of the structural changes induced by sIp in skeletal TnC (+15 helical residues) and cardiac TnC (-3 helical residues) do not involve residues which are involved in Ca^{2+} binding.

Ca^{2+} TITRATIONS OF THE TnI PEPTIDE-TnC COMPLEXES

In order to determine whether binding of the inhibitory peptides affects Ca^{2+} affinity of skeletal and cardiac TnC, pCa curves were obtained from Ca^{2+} titration of TnC

and TnC-peptide complexes. Two methods, CD and Tyr fluorescence, were used to monitor the Ca^{2+}-dependent changes. Since there is no Tyr in the amino acid sequences of the TnI inhibitory peptides, any change in the Tyr fluorescence on peptide or Ca^{2+} binding is due to changes in the environment of one or more Tyr residues located in TnC. In skeletal TnC there are two Tyr residues at positions 10 and 109 and in cardiac TnC there are three Tyr at positions 5, 111 and 150. Residue 109 of skeletal TnC and 111 of cardiac TnC are in the Ca^{2+} binding loop of site III. Previously, Johnson and Potter (1978) observed a biphasic increase in negative ellipticity (measured by CD) and Tyr fluorescence of skeletal TnC with increasing Ca^{2+} concentrations. The biphasic response was interpreted in terms of distinct changes in secondary structure in TnC when Ca^{2+} bound to the high and then low affinity Ca^{2+}-binding sites. The $pCa_{1/2}$ values obtained in this study for skeletal TnC when monitored by circular dichroism were 7.31 ± 0.09 and 5.72 ± 0.11 for the high and low affinity sites, respectively and 7.28 ± 0.06 and 5.42 ± 0.10 for the high and low affinity sites when monitored by Tyr fluorescence. These values are in excellent agreement with the $pCa_{1/2}$ values reported previously (Johnson and Potter, 1978). The $pCa_{1/2}$ values for cardiac TnC when monitored by circular dichorism were 7.17 ± 0.01 and 5.89 ± 0.11 for the high and low affinity sites and 7.02 ± 0.02 and 5.58 ± 0.11 for the high and low affinity sites when monitored by Tyr fluorescence. In the present study, the effect of various TnI inhibitory peptides on the Ca^{2+}-dependent conformational changes and the effect on the $pCa_{1/2}$ values of the high and low affinity sites of skeletal and cardiac TnC are examined (Figures 26 and 27).

TnI Peptide Effect on Skeletal TnC

Binding of sIp to skeletal TnC resulted in a leftward pCa shift of +0.30 units (Fig. 26, panel A, Table 15) which can be specifically attributed to a change in Ca^{2+} affinity at the low affinity Ca^{2+} binding sites I and II. Thus a lower Ca^{2+} concentration is required to fill the low affinity regulatory sites of TnC in the presence of sIp. Similarly, the change in

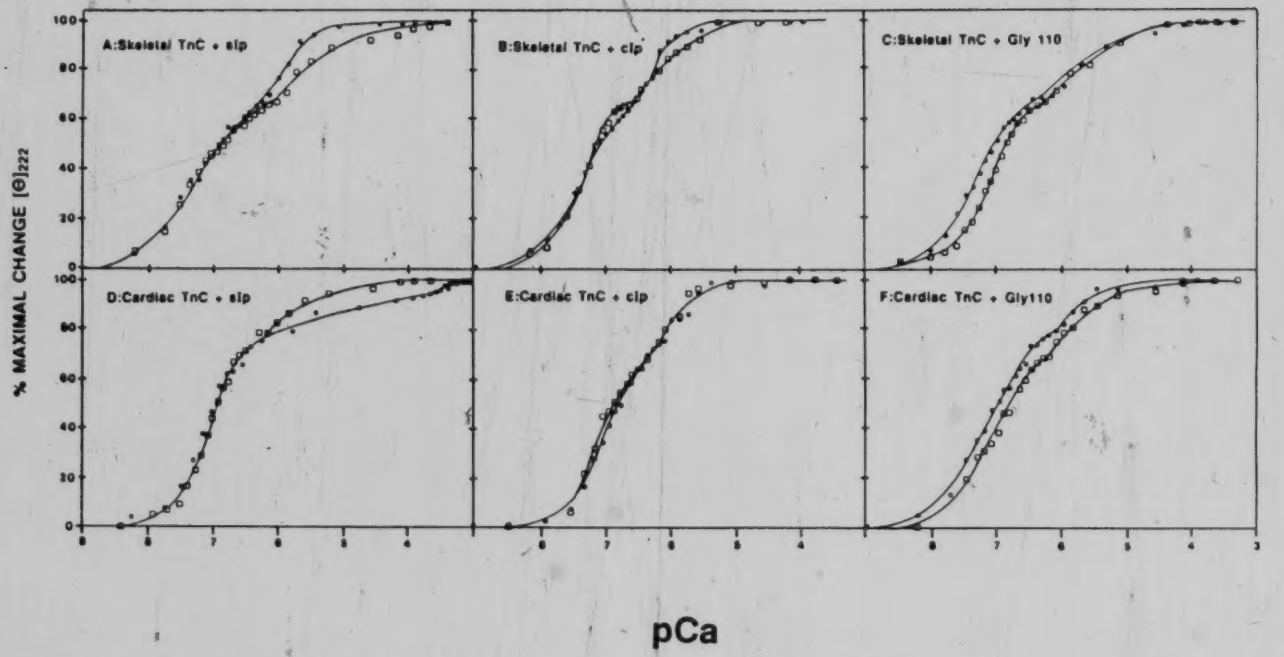

FIGURE 26: Effect of inhibitory peptides on Ca^{2+} binding to skeletal TnC and cardiac TnC monitored by circular dichroism. The % maximal change in ellipticity of TnC in the absence (open symbol) and presence (closed symbol) of sIp (panel A and D), cIp (panel B and E) and Gly 110 (panel C and F) are plotted versus pCa value. Top panels (A to C) show the effect of peptides on skeletal TnC while the bottom panels (D to F) show the effect of peptides on cardiac TnC. A 1:1 molar equivalent of various peptides and protein was used. The curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. Assays were performed as described in the methods section.

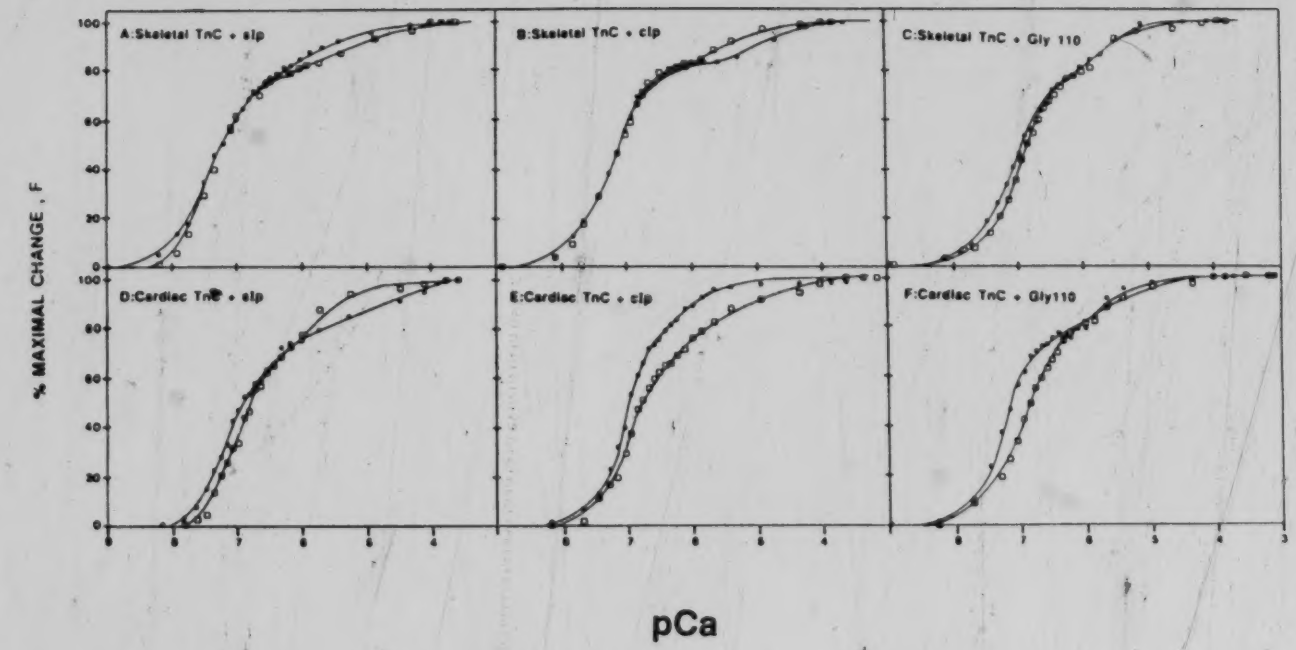

FIGURE 27: Effect of inhibitory peptides on Ca^{2+} binding to skeletal TnC and cardiac TnC monitored by Tyr fluorescence. The % maximal change in fluorescence of TnC in the absence (open symbol) and presence (closed symbol) of sIp (panel A and D), cIp (panel B and E) and Gly 110 (panel C and F) are plotted versus pCa values. Top panels (A to C) show the effect of peptides on skeletal TnC while the bottom panels (D to F) show the effect of peptides on cardiac TnC. A 1:1 molar equivalent of peptide to protein was used. The curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. Assays were performed as described in the methods section.

Table 15. Effect of TnI peptides on the $pCa_{1/2}$ values for the low and high affinity Ca^{2+} binding sites of skeletal and cardiac TnC

Method for Determination[1]	Peptide	ΔpCa^2 sTnC low affinity sites	sTnC high affinity sites	cTnC low affinity site	cTnC high affinity sites
CD	sIp	+0.30	NE[3]	-0.35	NE
	cIp	+0.30	NE	NE	NE
	Gly 110	NE	+0.21	+0.14	+0.14
F	sIp	+0.26	NE	-0.52	NE
	cIp	-0.42	NE	+0.58	NE
	Gly 110	NE	+0.16	NE	+0.30

1 CD stands for circular dichroism and F for Tyr fluorescence

2 The change in pCa (ΔpCa) was determined from the difference between the $pCa_{1/2}$ value of skeletal or cardiac TnC in the presence versus the absence of the peptide. A positive ΔpCa value indicates a leftward pCa_1 shift while a negative ΔpCa value indicates a rightward shift.

3 NE denotes no effect. If the peptide altered the $pCa_{1/2}$ value of sTnC or cTnC by 0.10 or less, it was considered to have no effect.

Ca^{2+} affinity was also monitored by fluorescence. sIp caused a leftward pCa shift (+0.26) at the low affinity sites indicating that the environment of at least one Tyr residue in skeletal TnC was altered in the presence of sIp. As well, cIp caused a pCa shift at the low affinity Ca^{2+} binding sites of skeletal TnC when monitored by either CD or fluorescence (Table 15). However, the direction and magnitude of the changes at the low affinity sites were different, depending on which method was used. The pCa shift was leftward (+0.30 units) when monitored by CD (indicating less Ca^{2+} was required to induce α-helix changes at the low affinity sites), while the pCa shift was to the right by -0.42 units when Tyr fluorescence was used (indicating a greater Ca^{2+} concentration was required, Table 15). It

would thus appear that the CD and fluorescence changes are independent of each other in that they monitor Ca^{2+} affinity at different low affinity Ca^{2+} binding sites. It is interesting that Gly 110 caused a pCa shift at the high affinity sites with no effect on the low affinity sites of TnC as indicated by CD and fluorescent monitoring.

TnI Peptide Effect on Cardiac TnC

The binding of sIp to cardiac TnC resulted in a major pCa shift at the low affinity Ca^{2+} binding sites (Fig. 26, panel B). When monitored by CD or Tyr fluorescence, the pCa shift was rightward by -0.35 units and -0.52 units, respectively (Table 15). The interaction between cIp and cardiac TnC caused a large leftward shift (+0.58 units) at the low affinity site when detected by Tyr fluorescence, but when monitored by CD there was no effect at either the high and low affinity sites. As discussed previously, the two methods can detect different structural changes in TnC (Fig. 27, panel A and B). The effect of cIp binding on the Tyr environment is Ca^{2+}-dependent, while the effect of the peptide on the α-helix structure is not sensitive to Ca^{2+}. Unlike the two native TnI peptides, Gly 110 caused pCa shifts at the high affinity sites in cardiac TnC when the pCa curves were determined by a change in fluorescence (Fig. 27, panel C). The leftward shift (+0.30 units) indicates that a lower Ca^{2+} concentration is required to change the environment of the Tyr which is sensitive to Ca^{2+} binding at sites III and IV (Table 15). On the other hand, CD detected only minor changes at the low and high affinity sites. Changes in the environment of Tyr residues in cardiac TnC are more sensitive to the structural changes in TnC upon peptide binding (especially cIp and Gly 110) than is the change in α-helical content as measured by CD.

DISCUSSION

It is not surprising that the TnI peptides affect Ca^{2+} binding, since the native protein, skeletal TnI, increases the Ca^{2+} binding affinity of TnC (Wang & Cheung, 1985;

Potter et al.,1976). It has been shown that the skeletal TnI peptide and other TnC-binding molecules like mastoparan and TFP increase the Ca^{2+} binding affinity of skeletal TnC (Van Eyk and Hodges, 1987, Rüegg et al., 1989; Cachia et al., 1983b). There is a discrepancy in the number of helical residues induced in skeletal TnC by the interaction with sIp. In Chapter 6 it was reported that sIp caused an increase of 7 and 13 helical residues in the absence and presence of Ca^{2+}, respectively, while in this chapter sIp caused an increase of 15 residues reguardless of the Ca^{2+} concentration. This discrepancy may be due to differences in the method used to prepare Ca^{2+}-free TnC in the various experiments (for more details see the method section). In the present work additional precautions were taken to ensure that the Ca^{2+}-free form of TnC was used. In the previous studies, it was not possible to differentiate whether the TnC-binding molecules were affecting the low or high affinity sites. The present work demonstrates for the first time that the binding of skeletal TnI peptide or cardiac TnI peptide affects Ca^{2+} affinity at the low affinity Ca^{2+} binding sites of skeletal or cardiac TnC, while the Gly 110 analog primarily affects the high affinity sites.

The changes in secondary structure which occur due to the binding of skeletal or cardiac TnI peptide to skeletal TnC affect the binding of Ca^{2+} at the low affinity regulatory sites (located in the N-terminal domain). This is of interest since experimental evidence has indicated that the major site of interaction between skeletal TnI peptide (104-115) and skeletal TnC is the N-terminal helix of Ca^{2+} binding site III (helix E) which is located in the C-domain of TnC (for a review see Cachia et al., 1983b). The interaction between TnI and residues 89-100 (helix E) in the C-terminal domain of TnC is well documented (Chong and Hodges, 1981; Grabarek et al., 1981; Dalgarno et al., 1982; Leavis & Gergely, 1984; Tao et al., 1986; Lesyzk et al., 1987). In fact, CNBr fragment of TnC (residues 83-134) forms a Ca^{2+}-dependent complex with TnI and is able to partially release TnI mediated inhibition of actomyosin ATPase activity and *in vitro* phosphorylation of TnI at Ser 117

(Weeks and Perry, 1978). Proteolytic fragments and synthetic peptides of the inhibitory region of TnI (minimum sequence residues 104-115), which is adjacent to Ser 117, mimic the activity of TnI in their ability to inhibit actomyosin ATPase activity and bind TnC resulting in the release of inhibition (Talbot and Hodges, 1979, 1981b; Katayama and Nozaki, 1982; Cachia et al., 1983a, 1986; Van Eyk and Hodges, 1987, 1988). It is important to point out that Cys 98 (helix E) of TnC is crosslinked specifically to residues within the inhibitory region of TnI whether in the IC or whole Tn complex (Lesyzk et al., 1987, 1988). In addition, crosslinking of a photoaffinity labelled TnI inhibitory peptide (benzophenone moiety attached to the α-amino group of the inhibitory peptide) to TnC results in the labelling of the C-terminal domain of TnC (sites III and IV) (Ngai and Hodges, unpublished data). Only a single crosslinking study (using TnC with its carboxyl groups activated with a carbodiimide and crosslinked to TnI) has shown that the TnI inhibitory region could be crosslinked to the N-terminal domain of TnC (Lesyzk et al., 1990). The very selective nature of the crosslinking reagent (requiring amino groups within crosslinking distance to the activated carboxyl groups) could have prevented crosslinking from occurring with the C-terminal domain, either because of distance constraints or intramolecular crosslinking. The authors (Lesyzk et al., 1990) state that their results do not negate the importance of the interaction in the C-terminal domain. Lan et al. (1989) indicate that the preferred binding site of synthetic TnI inhibitory peptide is on the C-terminal domain of calmodulin or TnC. In fact, the TnI inhibitory peptide (104-115) did not alter the fluorescence emanating from a probe on Met 25 of TnC but did alter the environment around Cys 98. Taken together, all of the above studies indicate that the primary interaction site for TnI peptide is the C-terminal domain of TnC, around Cys 98 in helix E. However, it is possible that a second weaker binding site for TnI peptide is located in the N-terminal domain of TnC exist. Further work is required to clarify the location and importance of this second binding site of the TnI inhibitory region on TnC.

Presently it is not clear how skeletal TnI peptide and, by analogy, the cardiac TnI peptide, when bound near helix E in the C-terminal domain, can alter the binding at the low affinity sites located in the N-terminal domain of skeletal TnC. There may be communication between the two halves of the TnC molecule, possibly via the central α-helix (D/E linker) which connects the N- and C-terminal domains of TnC (Herzberg and James, 1985; Sundaralingam et al., 1985). It has been well documented that Ca^{2+} and TnI binding to TnC induces α-helix, in particular, the E-helix of Ca^{2+} binding site III (Nagy et al., 1978; Nagy and Gergely, 1979; Reid et al., 1981). The increase in helical content is seen upon binding of mastoparan (Cachia et al., 1986) to TnC or TFP binding to a proteolytic fragment of the Ca^{2+} binding site III of TnC or a synthetic peptide comprised of helix E (Gariépy and Hodges, 1983). It is conceivable that helix E, when altered by TnI (and possibly TnI peptide) could affect the N-terminal domain of TnC via the D/E helix. In fact, Wang et al., (1990), using a TnC mutant, demonstrated that binding a cation to high affinity sites altered the environment around the amino acid at position 57 in the N-terminal domain. In addition, binding Ca^{2+} to the low affinity sites alters the environment around Cys 98 which is located in helix E (Grabarek et al., 1986; Rosenfeld and Taylor, 1985). In the crystal structure, the central helix is extended, producing an elongated structure. On the other hand, there is some evidence derived from low-angle X-ray scattering (Heidorn and Trewhella, 1988; Hubbard et al., 1988) and fluorescence energy transfer (Wang et al., 1987) suggesting a more compact structure of TnC in solution. Bending of the D/E helix would bring the N- and C-terminal domains into close contact, which would allow direct communication between the two domains. Hence, the TnI peptide when bound in the C-terminal domain of TnC could possibly interact with the N-terminal domain of TnC

SIp also altered the low affinity Ca^{2+} binding site of cardiac TnC, similarly to skeletal TnC. However, the pCa shift is toward higher Ca^{2+} concentrations. The proposed binding site on skeletal TnC (residues 88-102) is highly conserved in cardiac

muscle (residues 90-104) with 11 out of 15 residues identical. The effect of the three peptides on the secondary structure (when monitored by either CD or fluorescence) differs depending on whether the peptide is bound to skeletal or cardiac TnC. However, the two native peptides alter the Ca^{2+} affinity of the low affinity sites while Gly 110 also alters the high affinity sites of both TnC species. This finding indicates that although the conformational change differs depending on the species of TnC, which of the Ca^{2+} binding sites are affected is not dependent on the TnC species, but rather on which peptide is used. Therefore, the primary factor that dictates if the regulatory low affinity Ca^{2+} binding sites are altered by the binding of the TnI peptide, maybe the difference in amino acid sequence of the inhibitory region of TnI rather than the differences between skeletal and cardiac TnC.

The binding of cIp or Gly 110 produces more subtle changes in the secondary structure than those displayed by sIp, and these are detected more efficiently by the changes to the environment of Tyr. The single amino acid substitution between skeletal and cardiac TnI peptide of a Pro to Thr alters the effect of the TnI peptides on the secondary structure of skeletal and cardiac TnC, but not the ability of both peptides to affect Ca^{2+} binding at the low affinity sites. It is conceivable that the secondary structure adopted by the two TnI peptides is different, and the peptides may affect different amino acid residues on TnC, which, in turn, would alter the secondary structure of TnC differently. The $\theta_{200nm}/\theta_{222nm}$ ratio was not similar for cIp and sIp, indicating strongly that the conformations in solution and in the presence of TFE are vastly different. The structure of the skeletal TnI peptide bound to skeletal TnC in the presence of Ca^{2+} determined by NMR has the Pro residues forming a 'turn-like' structure so that the hydrophobic residues F 106, L 111 and V 114 are in close proximity (Chapter 5; Campbell and Sykes, 1991). It seems likely that the conformation adopted by cIp when bound to TnC is probably different from sIp. This suggestion is supported by TnC-affinity chromatography of the cardiac TnI analogs in which single amino acids are individually substituted with Gly. These analogs bound to

skeletal and cardiac TnC affinity columns with different strengths than the corresponding skeletal Gly analogs (Van Eyk and Hodges, unpublished data), suggesting that residues which are important for TnC binding are different for skeletal TnI 104-115 than those which are involved in the binding of cardiac TnI 137-148. Gly 110 should be more flexible than the other two TnI peptides, since Gly does not have a side chain and so can adopt a wider range of ψ and ϕ dihedral angles than Pro or Thr. Surprisingly, the $\theta_{200nm}/\theta_{222nm}$ ratios for Gly 110 and cIp are similar, but different from sIp. Since the peptide does alter the $pCa_{1/2}$ of the high affinity sites, it is clear that the Gly 110 analog alters the secondary structure of skeletal and cardiac TnC differently than the two native TnI peptides which affect mainly the low affinity sites. It is important to note that it is the low affinity sites which are the regulatory sites for muscle contraction. This difference in the affect of the Gly 110 analog compared to the two native peptides may be reflected in the fact that skeletal and cardiac TnI peptides induce equivalent inhibition of force development of skeletal and cardiac skinned muscle fibres, while Gly 110 does not significantly alter force development (Rüegg et al., 1991).

In conclusion, the TnI inhibitory peptides, upon binding cause differences, though sometimes subtle ones, in the structure of skeletal and cardiac TnC which, in turn, alter the Ca^{2+} affinities. This fact indicates that even though the two TnC molecules are highly conserved, they behave differently to TnI binding. As well, the three TnI peptides act differently from one another, indicating that the single amino acid change in inhibitory sequence is partly responsible for the differences in the biological activity of skeletal and cardiac muscle.

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CHAPTER 8

A SYNTHETIC PEPTIDE OF THE N-TERMINUS OF ACTIN INTERACTS WITH MYOSIN

INTRODUCTION

Immunological data, crosslinking, and NMR experiments have strongly indicated that the N-terminal region of actin contains one of the binding sites between actin and myosin (Sutoh, 1982,1983; Moir & Levine, 1986; Moir *et al.*, 1987; Mejéan *et al.*, 1986,1987; and Miller *et al.*, 1987). The recent actin structure determined by X-ray crystallography of the actin: DNAase I complex showed that regions within the N-terminal residues 1-32 of actin, in particular residues 1-7 and 20-28, are located on the surface of the actin molecule (Kabsch *et al.* 1990). In the actin structure and the proposed model of the F-actin filament, the N-terminal residues are exposed and not involved in either the DNAase-actin or the proposed actin-actin interactions (Holmes *et al.*, 1990). As well, the actin residues 1-7 have high temperature factors which indicate that this region is surface-exposed and mobile. Therefore, the biological activity of a synthetic peptide of actin residues 1-28 (Ac-D-E-D-E-T-T-A-L-V-A-D-N-G-S-G-L-V-K-A-G-F-A-G-D-D-A-P-R-amide) was examined in S1, acto-S1 and HMM ATPase assays and compared to F-actin. This work has been submitted to Biochemistry.

RESULTS AND DISCUSSION

ACTIN PEPTIDE 1-28 INTERACTION WITH S1

Complex formation between S1 and actin peptide 1-28, (in the absence of ATP) was determined by high performance size-exclusion chromatography (SEC) as shown in Figure 28. A pH value of 6.8 was chosen for SEC because the peptide-S1 complex is

soluble at this pH and shows no formation of a precipitate as observed at the higher pH values used in the bioassays (see below). Due to the large difference in molecular weight between S1 (150,000 daltons) and actin peptide (2,274 daltons), these components are easily resolved by SEC. The formation of a peptide-S1 complex is indicated by the presence of peptide in the S1 containing fractions (Figure 28, panel B and C). It is intriguing that the S1 and peptide-S1 complex were partially resolved and eluted in the opposite order to that expected based upon size considerations alone. Fraction 11 contained S1 only, while the fractions 12 and 13 contained both peptide and S1 (compare panels B and C). This result indicates that a conformational change in S1 has occurred upon peptide binding such that the peptide-S1 complex is more compact and hence, is eluted from the SEC column as if it had a smaller apparent molecular weight. RPC (reversed-phase chromatography) on a hydrophobic interaction column is ideal to monitor the fractions obtained from size-exclusion chromatography. The low ligand density on the hydrophobic interaction column allows an excellent separation between the actin peptide and S1 (A1, A2) (Figure 28, panel D). The S1 heavy chain and two light chains (A1, A2) could not be eluted from a standard reversed-phase column which has a ligand density approximately 10 times greater than the HIC column. The actin peptide does not bind to the HIC column under these conditions and is eluted just after the breakthrough salt peak (panel D).

S1 ATPase assays were examined to determine whether the actin peptide 1-28 interaction had any effect on the biological activity of S1. Unfortunately, mixtures of S1 and peptide formed a precipitate between pH 7.4 and 8.2 which is the pH range where S1 has optimal enzymatic activity. Therefore, centrifugation studies were done under these conditions to evaluate this interaction by comparing the ratio of peptide and S1 in the precipitate (in the absence and presence of ATP). In the absence and presence of ATP, the precipitate was composed of equivalent quantities of peptide and S1 at all ratios of peptide

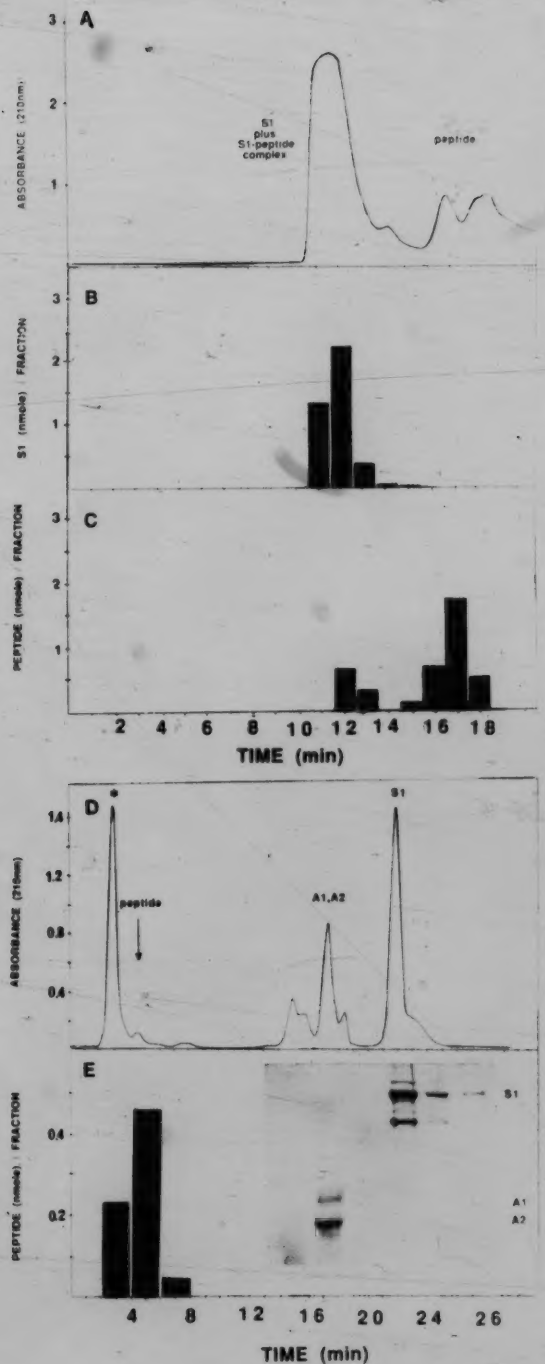
A
S1
plus
S1-peptide
complex
peptide
ABSORBANCE (210nm)
B
S1 (nmole) / FRACTION
C
PEPTIDE (nmole) / FRACTION
2 4 6 8 10 12 14 16 18
TIME (min)
D
S1
peptide
A1,A2
ABSORBANCE (210nm)
E
S1
A1
A2
PEPTIDE (nmole) / FRACTION
4 8 12 16 20 24 26
TIME (min)

Figure 28. Actin peptide 1-28 and S1 interaction determined by size-exclusion chromatography. Panel A shows the elution profile of a mixture of S1 and actin peptide at a 1:1 mole ratio (4.2 nmoles of each component). The chromatographic conditions are described in the method section. Fractions were collected and analyzed for the presence of S1 and peptide. S1 was identified and quantitated in the SEC fractions by reversed-phase chromatography on a hydrophobic interaction column as described in the method section (panel B). A representative separation of peptide, S1 heavy chain, and the A1, A2 light chains are shown in panel D. The * in panel D is the salt present from the SEC fraction (3 min), while the radiolabelled peptide is eluted at 5 min as detected in panel E. SDS urea gel electrophoresis results of each 2 minute fraction from RPC in the 14 to 28 minute section of the chromatogram (panel D) are shown in panel E (insert). The quantity of peptide present in each SEC or RPC fraction was determined by radioactivity measurements (panels C and E).

to S1 done in the assay (Figure 29, panel A). This result suggests that on formation of a 1:1 complex, the peptide-S1 complex is less soluble allowing aggregation and subsequent precipitation to occur. As expected, as the ratio of actin peptide to S1 increases, there is a linear increase in the amount of S1 precipitated (Figure 29, panels B and D). At a 1:1 ratio of S1 to peptide, in the absence of ATP, approximately 25% of the total S1 present in the assay is precipitated (Figure 29, panels B and D) while in the presence of ATP, 15% of the total amount of S1 precipitates (Figure 29, panel B). Even though low levels of precipitation of S1-peptide complex occur, the effect of the actin peptide on the S1-ATPase was examined (Figure 29, panel C). Under these conditions, the actin peptide 1-28 potentiates S1-ATPase activity from 0.032 s^{-1} in the absence of peptide to 0.057 s^{-1} in the presence of peptide. The maximum ATPase rate occurs at an approximate 1.5:1 mole ratio of peptide to S1.

To determine whether the S1-peptide complex in the precipitate and supernatant was biologically active, individual tubes of S1, in the absence of ATP, with different quantities of peptide were prepared. After centrifugation, the supernatant was removed and the ATPase activity of the supernatant was determined by addition of ATP. As shown in Figure 29, panel C, the ATPase activity of the S1-peptide in the supernatant was less than ATPase activity where the precipitate was suspended (closed versus open circles, respectively). The activity of the suspension was the same whether increasing quantities of

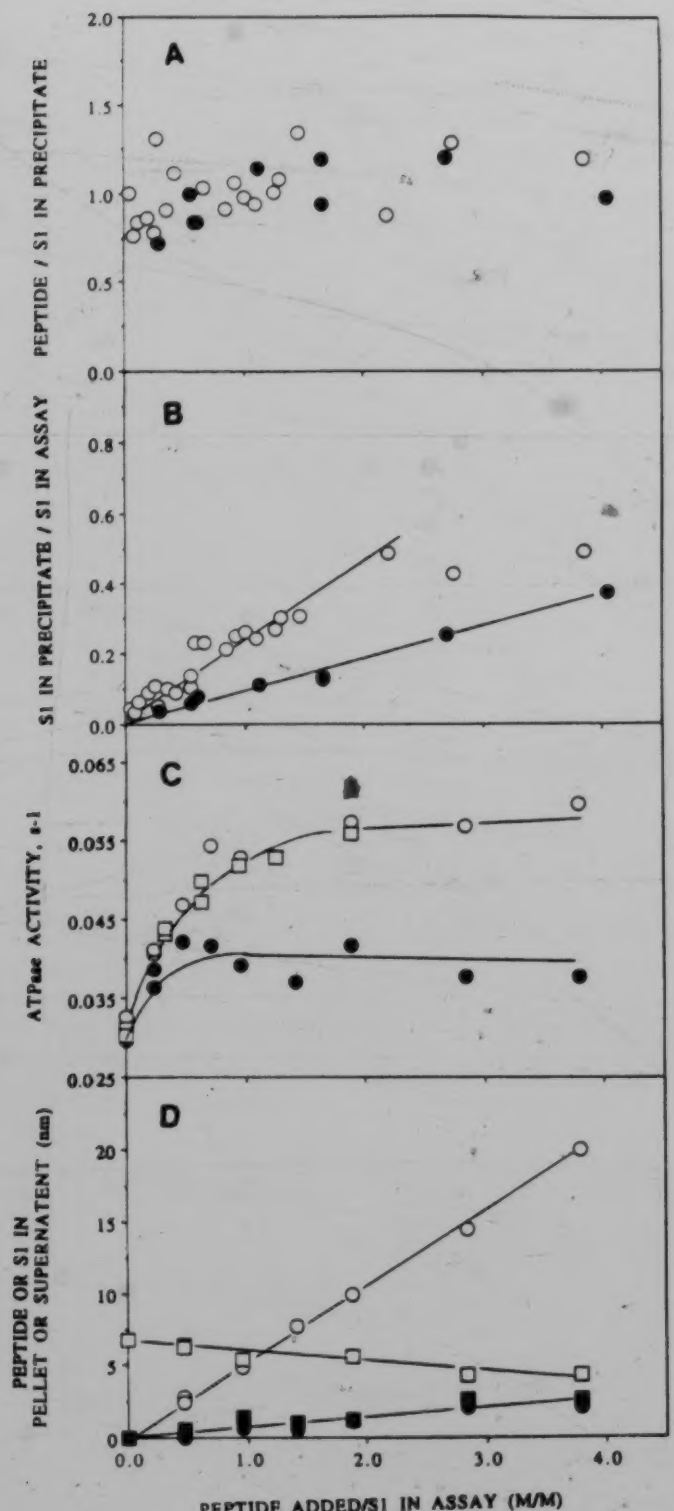
A
B
C
D
PEPTIDE / S1 IN PRECIPITATE
S1 IN PRECIPITATE / S1 IN ASSAY
ATPase ACTIVITY, s-1
PEPTIDE OR S1 IN PELLET OR SUPERNATENT (nm)
PEPTIDE ADDED/S1 IN ASSAY (M/M)
2.0
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
0.065
0.055
0.045
0.035
0.025
20
15
10
5
0
0.0
1.0
2.0
3.0
4.0

Figure 29. Interaction between actin peptide 1-28 and S1 in the absence and presence of ATP. Centrifugation studies were carried out in a mixture of 4 or 5.8 nmoles of S1 (A1,A2) in the absence of ATP and 4 nm of S1 (A1,A2) in the presence of ATP plus increasing quantities of actin peptide. Assay conditions are described in the Method section. After centrifugation, aliquots of the supernatant were analyzed for S1 and peptide. From this data the ratio of peptide to S1 in the absence (○) and presence (●) of ATP in the precipitate was determined (panel A). The amount of S1 in the precipitate formed as a ratio of total S1 in assay in the absence (○) and presence (●) of ATP when increasing quantities of peptide were added is shown in panel B. The effect of the actin peptide on S1 ATPase activity is shown in panel C. The concentration of S1 is 2.9 μM and assay conditions are described in the Method section. Increasing quantities of peptide were added to a single vial of S1 such that the S1-peptide precipitate was suspended in the assay solution following the continuous titration method (□). As well, three identical sets of tubes containing mixtures of S1 and different quantities of peptide were centrifuged at 15,000 rpm for 15 minutes at 4°C to pellet the precipitate. From one set of tubes, the supernatant was removed and the ATPase activity determined by addition of ATP (●). The ATPase activity (s^{-1}) of the supernatent was determined based on the concentration of S1 in the supernatent as determined in panel D. The second set of tubes were vortexed to resuspend the pellet back into the supernatent (○). The ATPase activity (s^{-1}) for this resuspended sample was determined based on the S1 concentration originally added and still present in the assay. The resuspended samples were assayed by addition of ATP. Panel D shows the corresponding quantities of peptide (circle) and S1 (square) located in the pellet (closed symbol) and supernatant (open symbol) at each specific quantity of peptide which were determined using the third set of tubes (following the method used in panels A and B).

peptide were added to a single vial containing S1, or to individual tubes of S1 to which varying quantities of peptide were added, centrifuged and then vortexed to resuspend the pellet indicates that centrifugation of the samples had no effect on the results (open squares versus circles, respectively). The fact that the ATPase activity of the supernatant was less than when the pellet was suspended in the supernatant implies that S1-peptide complexes in both the pellet and supernatant are active. Figure 29, panel D shows the amount of S1 and peptide present in the pellet and supernatant after centrifugation and prior to addition of ATP. It is significant that the S1-peptide complex located in the pellet increased with increasing addition of peptide even after the ATPase activity had reached a maximum rate. This further confirms that the S1-peptide complex located in the pellet must be active.

Since the S1-actin peptide complex precipitated in the S1-ATPase assay, we concluded that this assay is inappropriate for detailing quantitatively the interaction of the peptide and S1. However, it did demonstrate that the peptide binds to S1 and is able to

activate ATPase activity. It is preferable to find assay conditions where no precipitation occurs (see below).

ACTIN PEPTIDE 1-28 INTERACTION WITH ACTO-S1 AND HMM

Two alternative assays were used to measure the effects of actin peptide on myosin ATPase activity under conditions where no precipitation occurred. First a larger myosin fragment, HMM, was employed instead of S1; and second, F-actin was added to the S1-ATPase assay prior to the titration with the actin peptide. The actin peptide did not precipitate S1 in the presence of actin or HMM. Figure 30, panel B compares the activation of HMM ATPase activity by the actin peptide and F-actin. At low concentrations of peptide (below 0.5:1 mole ratio of peptide to HMM), the peptide was as effective as F-actin and increased the ATPase activity by 62%. At higher concentrations (above a 0.5:1 mole ratio of HMM), F-actin continued to activate the ATPase rate while the peptide had reached its maximum activation. This suggests that F-actin filament formation may be required for the increased activation of HMM ATPase activity above the 0.5:1 ratio of peptide to HMM as seen with F-actin-HMM. Since actin is filamentous, it may not act the same as monomeric actin. There have been numerous studies suggesting that the binding of S1, and presumably HMM, to the F-actin filament induced long range conformational changes along the filament (Ikkai *et al.*, 1979, Yanagida *et al.*, 1984 and Rouayrenc *et al.*,1985). As well, in skinned muscle fibres there appears to be cooperativity within a regulatory unit, which consists of 7 actin and 1 TM-Tn, that causes an increase in force induction (Bremel and Weber 1972; Brandt *et al.*, 1984; Moss *et al.* 1985, 1986). These results suggest that the binding of myosin or a myosin fragment to actin is influenced by the remaining unbound actin in the filament resulting in the alteration of the ATP hydrolysis rate. The increase in ATPase activity induced by the F-actin filaments at high ratios may reflect the cooperativity within the F-actin filament, while at low concentrations, reflect the binding and influence of a single actin molecule.

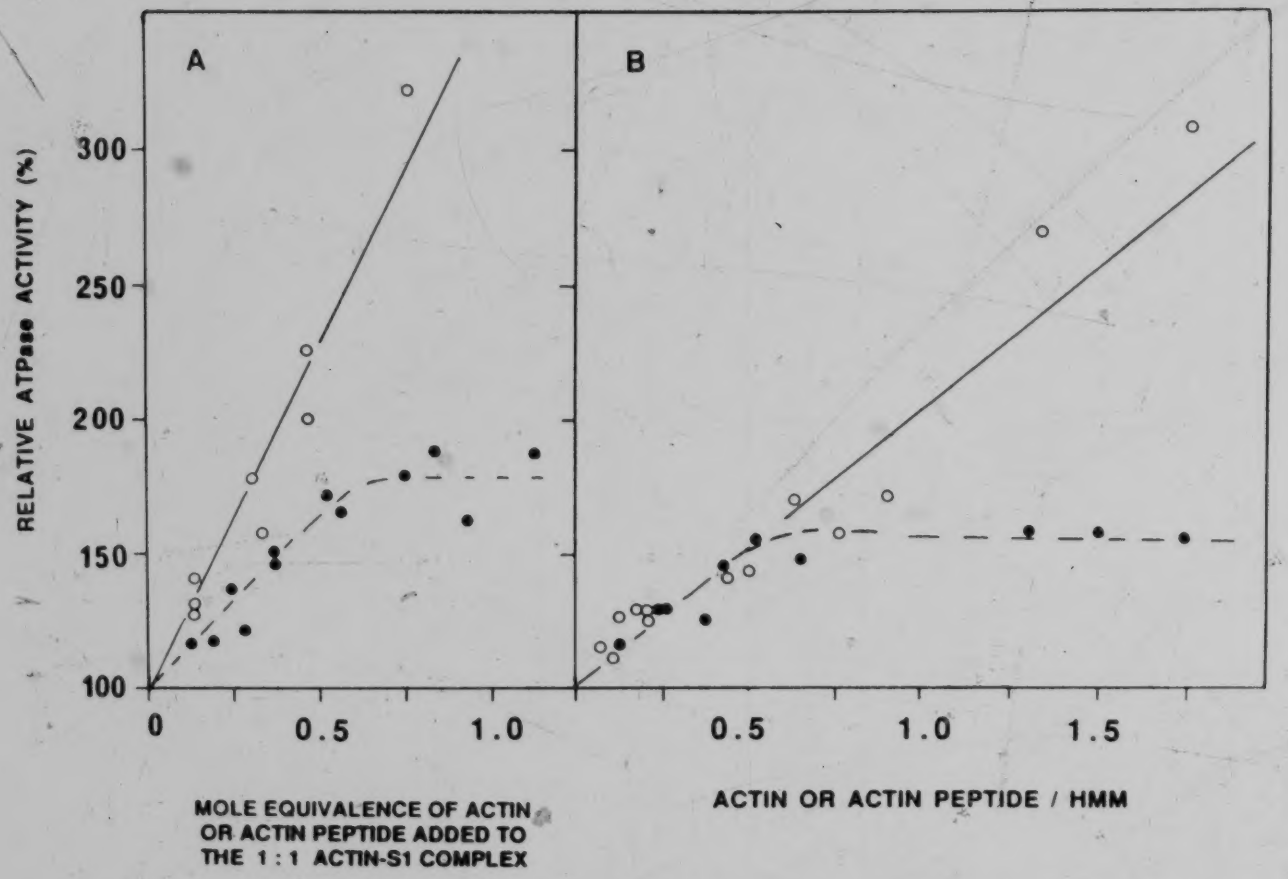

Figure 30. The effect of F-actin and actin peptide 1-28 on the acto-S1 (panel A) and HMM (panel B) ATPase activities. The acto-S1 ATPase activity in the absence of actin peptide or additional F-actin was taken to be 100%. The concentration of actin and S1 were both 3 μM. The HMM ATPase activity in the absence of peptide or F-actin was taken to be 100%. The concentration of HMM was 2 μM. The assay conditions are described in the method section. The symbols are: F-actin (○) and actin peptide (●).

The effect of the actin peptide and F-actin on the acto-S1 ATPase activity is similar to its effect on HMM (Figure 30, panel A). The starting conditions for the acto-S1 assay consist of a 1:1 molar ratio of F-actin to S1 to which increasing concentrations of either F-actin or actin peptide (1-28) are added. The actin peptide is able to increase the acto-S1 ATPase activity in a manner similar to that of F-actin. At a 0.5:1 mole ratio of additional F-actin or actin peptide to S1, there is a 130 and 65% increase in the ATPase activities, respectively. At a 1:1 mole ratio of additional F-actin or peptide, F-actin causes a 260 % increase in the ATPase activity while the actin peptide has reached its maximum activity (75% increase). The maximum activity obtained by the peptide in the presence of 1 equivalent of F-actin was approximately 0.5 equivalents of peptide to S1 (Fig. 30, panel A) for a total 1.5:1 equivalents of the 1-28 segment to S1. This ratio of 1.5 equivalents of 1-28 to S1 required for maximum activity is identical to that obtained when adding the actin peptide alone to S1 (Figure 29, panel C).

When F-actin was present in the S1-ATPase assay, there was no precipitation of peptide-S1 complex at low ratios of peptide to S1. There are two possibilities that would explain this decrease in peptide - S1 precipitate. First, the F-actin bound S1, decreasing the concentration of S1 in solution available to interact with the peptide. Therefore, at any given time there would be less S1-peptide complex in solution, which would reduce the opportunity for aggregation of the peptide-S1 complex to occur. Second, it is possible that the peptide and F-actin could bind to S1 simultaneously resulting in a complex with increased solubility. To determine whether the actin peptide would bind simultaneously to the acto-S1 complex, centrifugation studies were performed. Increasing quantities of radiolabelled peptide were added to vials containing F-actin and S1. Minimal amounts of peptide were found in the acto-S1 pellet, in fact, the same quantity of peptide that was found in the F-actin pellet in the absence of S1 (Table 16). It is clear that peptide does not bind to the acto-S1 complex and does not form a complex of peptide-S1-actin. Therefore, when F-actin is present in the S1 ATPase assay (Figure 30, panel A), addition of the actin

peptide binds to S1 free in solution and not the S1 bound to F-actin. Thus, the activation of the ATPase activity observed upon addition of peptide is over and above the activation already induced by binding of F-actin to some of the S1.

ATPase data obtained from the actin peptide titrations of acto-S1 and HMM assays were analyzed by Michaelis kinetics to determine the concentration of peptide required to induce 50% of the maximum activation (K_{ATPase}) and the maximum activity (V_{max}) (Figure 31). The actin peptide had the same K_{ATPase} of 0.4 $x10^{-6}$ M and V_{max} of between

Table 16: Determination of the binding of actin peptide 1-28 to actin and the acto-S1 complex

Peptide added (nmole)	Amount of peptide in pellet/S1 in pellet		
	Actin[a]	Acto-S1[b] -ATP	Acto-S1[b] +ATP
0	0.00	0.00	0.00
1.0	0.04	0.03	0.07
2.1	0.09	0.13	0.05
2.9	0.12	0.25	0.14
3.8	0.20	0.17	0.25
5.9	0.16	0.17	0.20
11.1	0.29	0.17	0.17
22.5	0.38	0.34	0.23

a 5.1 nmole of F-actin was pelleted in the absence of S1 and peptide, hence there was 0.5 nm of F-actin remaining in the supernatant. These assays act as a control to determine background levels of peptide pelleted.

b 5.1 nmole of F-actin and S1 was pelleted in the absence of peptide, hence there was virtually no S1 remaining in the supernatant.

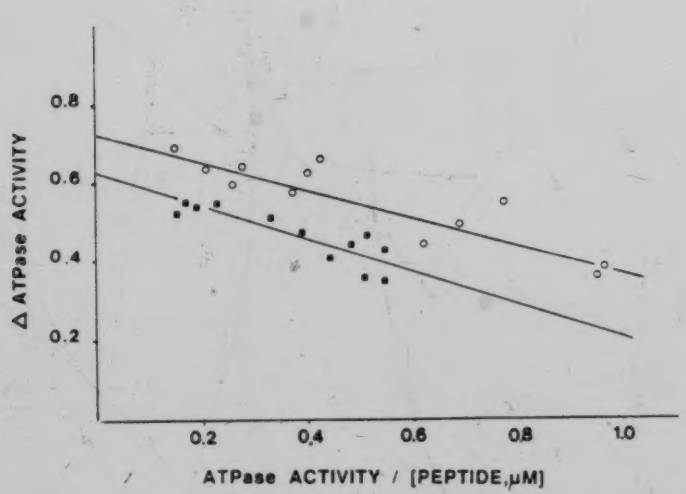

Figure 31. Analysis of the acto-S1 and HMM activation by the actin peptide 1-28. The experimental data for the actin peptide in either the acto-S1 (o) or HMM ( ) enzyme systems was plotted as V (change in ATPase activity, Δ ATPase activity) compared to V/[s] (% ATPase activity/actin peptide concentration in μM). The y intercept is the V_{max} and slope of the best fit line through the data points is equal to K_{ATPase}.

Table 17: Apparent binding constant and maximum activation of actin peptide 1-28 for S1 and HMM

Enzyme	K_{ATPase} (x 10^{-6} M)	V_{max}[a] (% activation)
Acto-S1	0.39 ± 0.05	173 ± 3
HMM	0.42 ± 0.09	162 ± 9

[a] V_o is equal to 100%. Therefore, V_{max} is the increase in the ATPase activity above V_o.

162-173%, for both enzyme systems (Table 17). Under similar experimental conditions, the actin-activated HMM or actin-activated S1 ATPase activity has an apparent binding constant of approximately 1 x 10^{-5} M (Stein *et al.*, 1979 and 1981). However, the V_{max} / K_{ATPase} of F-actin (at a 7:1 mole ration of F-actin to S1) and the actin peptide 1-28 is similar.

COMPETITION BETWEEN THE ACTIN PEPTIDE AND F-ACTIN

To investigate whether the actin peptide is able to compete with F-actin for S1, a series of peptide titrations was carried out at various F-actin concentrations (Figure 32, panel A). Both the actin peptide and F-actin functioned as activators of the S1 ATPase activity. At high concentrations of F-actin, the actin peptide was more effective at stimulating the S1-ATP hydrolysis rate, indicating that the presence of F-actin somehow acts to enhance the peptide's effect on S1 ATPase activity. A Lineweaver-Burke plot of $1/_{ATPase}$ activity versus either 1/peptide or 1/F-actin concentration (Figure 32, panels B & C, respectively) showed that both F-actin and peptide affect the V_{max} and K_{ATPase} of each other's activation of S1 ATPase activity. The slope and y intercept terms, obtained from the double reciprocal plots when plotted against the second activator, were hyperbolic (Figure 32, panels B & C, insert). The fact that the slope and y intercept terms are nonlinear in nature, indicates that both the F-actin and peptide are complex nonessential activators (Segal, 1979).

CONCLUSION

The synthetic actin peptide 1-28 activates the S1 ATPase activity by approximately 65%. Under conditions used in the ATPase assays, actin peptide and S1 precipitate as a 1:1 complex. However, approximately 15 to 25% of the total S1 in the assay had precipitated at 1 equivalent of peptide to S1. The actin peptide 1-28 did not precipitate HMM or acto-S1. The actin peptide caused the HMM and acto-S1 ATPase to be increased

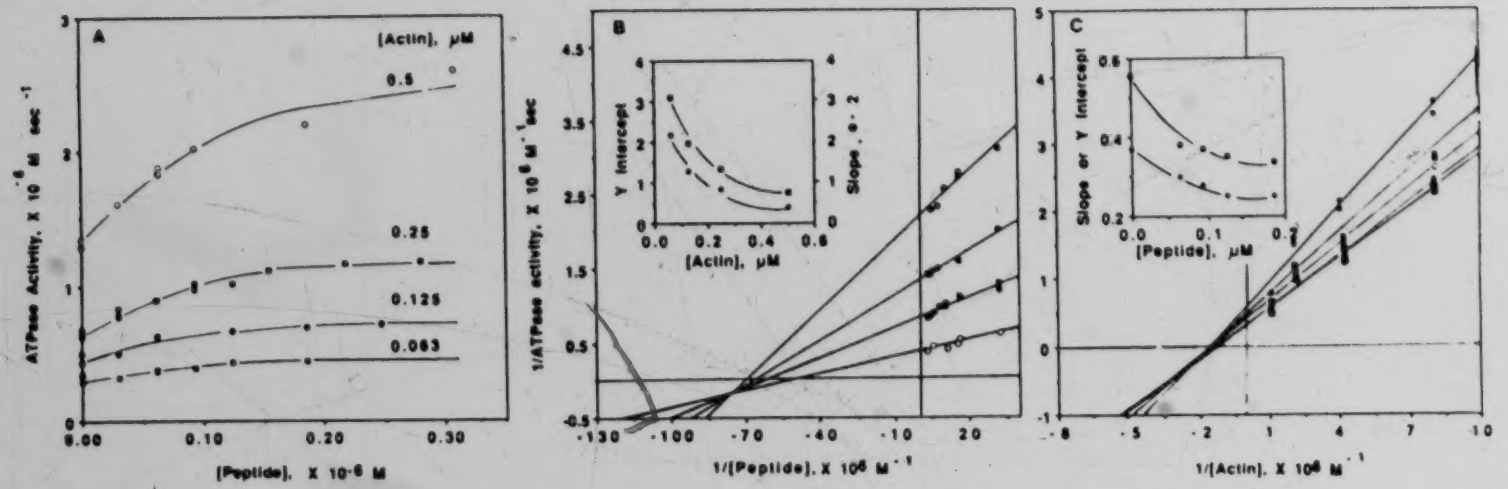

Figure 32. Competition results of F-actin and actin peptide in the S1 ATPase assay. The effect of the actin peptide on the S1 ATPase activity in the presence of 4 concentrations of F-actin; 0.063 (□), 0.125 (●), 0.25 (■) and 0.5 (○) μM are shown in panel A. The concentration of S1 was 1 μM. The Lineweaver-Burke plot of the effect of the peptide on the ATPase activity at four F-actin concentrations are shown in panel B while the Lineweaver-Burke plot of the effect of F-actin on the ATPase activity at five concentrations of peptide; 0 μM (●), 0.625 (■), 0.85 (○), 1.25 (▲), and 1.85 (□) are shown in panel C. The corresponding plots of the slope and y intercept terms versus concentration of F-actin or peptide are shown in panels B and C (inserts). The (●) and (○) symbols denote the slope and y intercept terms, respectively.

in a manner similar to F-actin at concentrations below 0.5 equivalence of HMM or 1.0 equivalence of S1. At higher concentrations, the peptide-induced activation levelled off, while F-actin continued to activate the ATPase rate.

These results indicate that the actin peptide can mimic in part, the biological activity of F-actin. Hence, the actin peptide must represent a biologically important binding site between actin and myosin.

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CHAPTER 9

SYNTHETIC PEPTIDE STUDIES ON THE INTERACTION OF THE N-TERMINAL REGION OF ACTIN WITH MYOSIN SUBFRAGMENT 1 (S1) AND HEAVY MEROMYOSIN (HMM)

INTRODUCTION

A synthetic peptide of actin, actin 1-28, causes a 1.5 fold activation of the S1 ATPase activity, while causing a 1.65-1.70 fold increase in the heavy meromyosin (HMM) and acto-S1 ATPase activities (Van Eyk and Hodges, 1991, Chapter 8). Notably, there is little difference between the F-actin and actin peptide activation of S1 and acto-S1 at mole ratios below 1-1.5 equivalent of peptide to S1 or 0.5 equivalent of peptide to HMM. At higher ratios of F-actin or actin 1-28 to S1 or HMM, only F-actin continued to activate the ATPase activities in a concentration-dependent manner. In order to determine the sequence required for biological activity and the relative importance of regions within actin 1-28 for the interaction with the myosin fragments, a series of nine truncated actin analogs were synthesized by solid-phase peptide methodology (Table 18).

RESULTS

A series of deletion analogs of the actin 1-28 were synthesized to delineate the essential regions within actin 1-28 sequence required to bind myosin and activate the ATPase activity (Table 18). The cysteine at position 10 in the native protein was substituted with an alanine to eliminate problems due to oxidation of the peptides. In peptides containing residue 1, the amino terminal amino acid was acetylated, as found in the native protein. As well, all peptides composed of internal sequences from actin were acetylated and amidated at the N- and C-terminus respectively, to eliminate

Table 18 Amino Acid Sequence of N-terminus of Actin and the Synthetic Actin Peptides

Actin Sequence	
	Ac D E D E T T A L V C D N G S G L V K A G F A G D D A P R
Peptide Sequence	
1-28	Ac D E D E T T A L V A D N G S G L V K A G F A G D D A P R - amide
1-24	Ac D E D E T T A L V A D N G S G L V K A G F A G D - amide
1-20	Ac D E D E T T A L V A D N G S G L V K A G - amide
1-14	Ac D E D E T T A L V A D N G S - amide
1-7	Ac D E D E T T A - amide
4-28	Ac E T T A L V A D N G S G L V K A G F A G D D A P R - amide
8-28	Ac L V A D N G S G L V K A G F A G D D A P R - amide
18-28	Ac K A G F A G D D A P R - amide
4-20	Ac E T T A L V A D N G S G L V K A G - amide
8-20	Ac L V A D N G S G L V K A G - amide
Peptide Sequence with position 4, 5 and 12 altered	
4-28 (D^4,E^5,D^{12})	Ac D E T A L V A D D G S G L V K A G F A G D D A P R
8-28 (D^{12})	Ac L V A D D G S G L V K A G F A G D D A P R

extra charges which are not present in the native protein (Talbot and Hodges, 1981).

THE EFFECT OF ACTIN ANALOGS ON THE S1-ATPase ACTIVITY

The effects of the various deletion actin analogs on the S1 ATPase activity are shown in Figure 33. Removal of amino acid residues from either the N-, C- or both N- and C-terminus of actin 1-28 (panels A, B and C) dramatically altered the S1 ATPase activity. Only actin 1-28 and actin 4-28 activated the S1 ATPase activity; other deletion analogs had either no effect or inhibited the ATPase activity. Actin 1-28 is the most effective and potent activator of the peptide analogs investigated, suggesting that the whole N-terminal region of actin is required for full biological activity. The effect of an analog on the ATPase activity is a function of its relative dissociation constant (K_{ATPase}) and maximum velocity (V_{max}) (Table 19).

The K_{ATPase} and V_{max} for each analog were obtained in a manner similar to analysis (assuming Michaelis kinetics) of the affects of TnI peptide analogs on the inhibition of the acto-S1 ATPase activity (Van Eyk and Hodges, 1988). The ATPase activities used in these calculations were obtained by subtracting the S1, acto-S1 or HMM ATPase activities in the absence of peptide (V_o) from the activity in the presence of peptide. V_{max} quantitates the efficiency of an analog to activate (or inhibit) the S1 ATPase at saturating concentrations of the analog. The lower the V_{max} for an analog which activates the ATPase rate, the less efficient the analog is at inducing the conformational changes in S1 required to increase the ATP hydrolysis rate (compare 1-28 and 4-28, Table 19, Figure 33, Panel B). On the other hand, if an analog inhibits the ATPase rate, then once the analog is bound to S1 it causes a detrimental effect on the conformation of S1 such that the ATP hydrolysis rate is decreased. The affinity of the analog for S1(K_{ATPase}) can be determined for analogs that activate or inhibit the ATPase activity. In either case, the larger

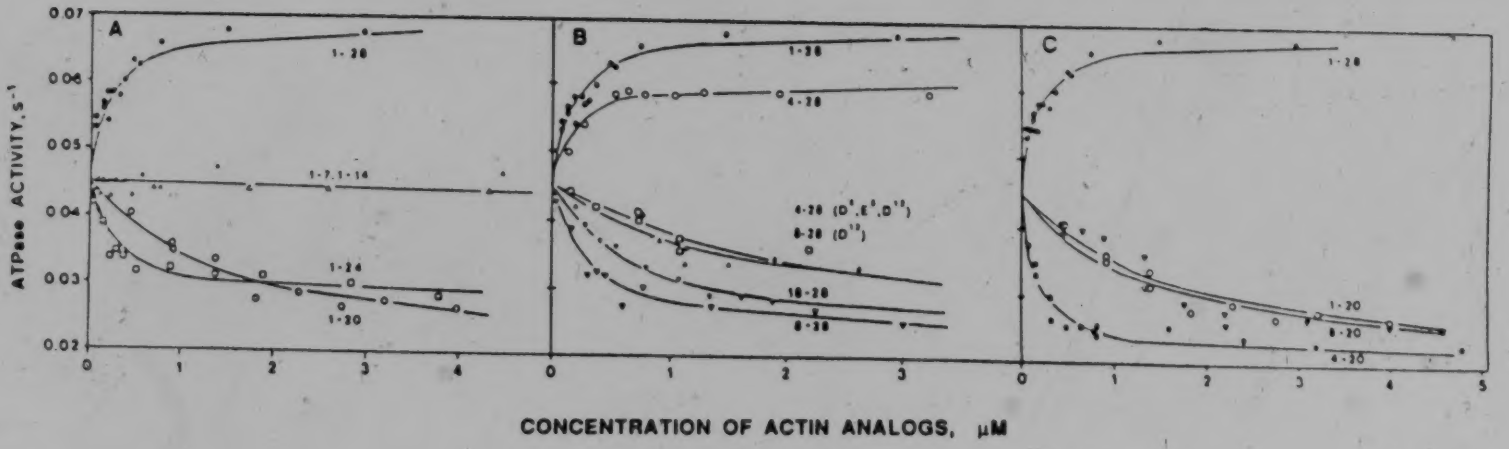

Figure 33. The effect on S1 ATPase activity by C-terminal (panel A), N-terminal (panel B) and C- and N-terminal truncated (panel C) actin 1-28 analogs. S1 ATPase rate was 0.045 s^{-1} in the absence of peptide. Assay conditions are described in the Method section. There was 1.75 μM S1 present in the assay. Symbols for actin peptides are as follows: 1-28 (•), 1-24 (□), 1-20 (○), 1-14 (▲), 1-7 (△), 4-28 (○), (D^4E^5,D^{12}) 4-28 (□), 8-28 (▽), (D^{12}) 8-28 (▲), 18-28 (▼), 4-20 (■) and 8-20 (▽).

Table 19. Analysis of S1 ATPase activity by actin peptides

Peptide	Type of Activity	K_{ATPase}[a] uM	V_{max}[b] s^{-1}	V_{max}/V_o[c]
1-28	Activation	0.14±0.02	0.068±0.001	1.51
4-28	Activation	0.14±0.04	0.060±0.001	1.33
1-14	No Effect			
1-7	No Effect			
1-24	Inhibition	0.22±0.04	0.028±0.001	0.62
1-20	Inhibition	1.21±0.20	0.021±0.001	0.47
4-20	Inhibition	0.15±0.02	0.022±0.001	0.49
8-20	Inhibition	1.63±0.36	0.019±0.002	0.42
4-28 (D^4,E^5,D^{12})	Inhibition	0.93±0.13	0.034±0.001	0.76
8-28	Inhibition	0.25±0.04	0.024±0.001	0.53
8-28 (D^{12})	Inhibition	1.27±0.63	0.026±0.005	0.58
18-28	Inhibition	0.69±0.09	0.023±0.001	0.51

[a] The K_{ATPase} for each analog was determined as described in the methods section.
[b] The initial velocity in the absence of peptide at a S1 concentration of 1.75 μM was 0.045 s^{-1}.
[c] The initial S1 ATPase rate was taken to be V_o.

the value of K_{ATPase} of a specific analog, the weaker the affinity between the analog and S1 and, hence, the residues that were deleted are important in binding this region of actin to S1.

The deletion of amino acid residues 1-3 from the N-terminus of actin 1-28 resulted in a small decrease in V_{max} (1.51 *vs* 1.33 for actin 1-28 and 4-28, Table 19). This suggests residues 1, 2 and 3 contribute marginally to the activation of the S1 ATPase activity. On the other hand, further deletion of residues 4 to 7 (actin 8-28) dramatically altered the biological properties of S1 since actin 8-28 inhibits the ATPase activity. This indicates that the N-terminal residues are critical in causing the changes in S1 that are responsible for activating the ATPase activity. However, this region is not important for binding since deletion of residues 1-7 increased the K_{ATPase} less than 2-fold [compare actin 8-28 (0.25 μM)to actin 1-28 or 4-28 (0.14 μM)]. It is interesting that the substitution analog (D^4, E^5, D^{12}) actin 4-28 inhibited the S1 ATPase activity, whereas actin 4-28 of the native sequence activated the ATPase activity. The deletion of amino acid residues 25 to 28 from the C-terminus of actin 1-28 (actin 1-24) also caused inhibition of the S1 ATPase activity with only a small decrease in the affinity of the peptide for S1 (K_{ATPase} was 0.22 μM for 1-24 and 0.14 μM for 1-28, Table 19 and Figure 33, Panel A). Therefore, the C-terminal region is critical for activation of the ATPase activity. Further deletion of residues 21 to 24 (actin 1-20) caused a large increase in the dissociation constant (1.21 μM for actin 1-20 compared to 0.22 μM for actin 1-24).

It is intriguing that the synthetic peptides, actin 1-7 and 1-14, show no effect on the S1 ATPase activity. Like actin 1-28 (Van Eyk and Hodges, 1991), all the actin analogs except 1-7, precipitated S1 in a 1:1 complex (determined by centrifugation studies, data not shown). The amount of precipitate varied from none for actin 1-7 to as high as 20% for actin 1-28 at a 1:1 mole ratio of peptide to S1. It was shown previously that the S1-actin

peptide 1-28 complex that precipitated was active and contributed to the observed ATPase activity. Therefore, the precipitation of peptide-S1 complex should not qualitatively alter the interpretation of the results of the various peptide effects on the S1 ATPase activity. However, it is clear that the interaction between the analogs and S1 causes a conformational change in S1 resulting in aggregation of the peptide-protein complex. In other words, the conformation of S1 is very sensitive to the binding of an actin peptide. The conformation of S1 is also sensitive to the binding of F-actin which results in activation of the ATPase activity. Therefore, it is possible that the various deletion actin analogs induce different conformational changes in S1 from one another and hence could have either a positive or negative affect on the S1 ATPase activity.

THE EFFECT OF ACTIN ANALOGS ON THE ACTO-S1 ATPASE ACTIVITY

In order to examine the ability of the actin peptides to compete with F-actin, the effect of the synthetic peptide analogs on the acto-S1 ATPase activity was determined. Starting conditions for the acto-S1 assay consisted of a 1:1 molar ratio of F-actin to S1 to which increasing concentrations of actin peptide were added. Van Eyk and Hodges (1991) have shown that the presence of F-actin in the S1 ATPase assays eliminates any precipitation of the peptide-S1 complex and that the peptide does not bind simultaneously to F-actin-S1 complex but rather competes with F-actin for S1. Thus, at any given time there would be less S1-peptide complex in solution, which would reduce the opportunity for aggregation of the peptide-protein complex to aggregate. Hence, K_{ATPase} determined in the presence of F-actin does not represent the true dissociation constant of the peptide-S1 interaction, since the analogs must compete with residues 1-28 of the native protein for S1. Instead K_{ATPase} reflects the ability of the peptide to bind S1, and its ability to compete successfully with F-actin. Table 20 lists the K_{ATPase} and V_{max} values of the various actin analogs obtained from the acto-S1 ATPase assays.

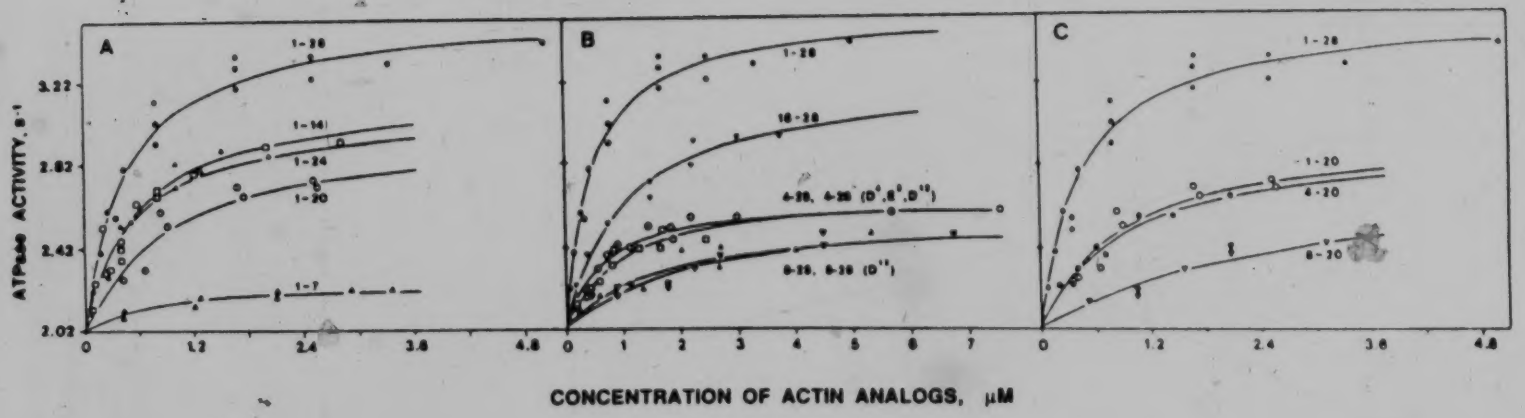

Figure 34. The effect on acto-S1 ATPase activity by C-terminal (panel A), N-terminal (panel B) and C- and N-terminal truncated (panel C) actin 1-28 analogs. Acto-S1 ATPase rate was 2.02 s^{-1} in the absence of peptide. Assay conditions are described in the Method section. The concentration of actin and S1 were each 2.5 μM . Symbols for the actin peptides are as follows: 1-28 (•), 1-24 (□), 1 -20 (○), 1-14 (▲), 1-7 (△), 4-28 (○), (D^4,E^5,D^{12}) 4-28 (□), 8-28 (▽), (D^{12}) 8-28 (▲), 18-28 (▼), 4-20 (■) and 8-20 (▽).

Table 20. The analysis of ATPase activation by actin peptides

Enzyme	Actin Peptide	K_{ATPase} (uM)	V_{max} (s^{-1})[a]	V_{max}/V_o[b]
A. Acto-S1	1-28	0.46±0.05	3.57±0.04	1.77
	1-24	0.48±0.08	3.05±0.06	1.51
	1-20	0.83±0.20	2.96±0.08	1.47
	1-14	0.59±0.09	3.15±0.06	1.56
	1-7	0.75±0.23	2.25±0.02	1.11
	4-20	0.91±0.11	2.93±0.04	1.45
	8-20	3.01±0.51	2.87±0.07	1.42
	4-28	0.64±0.09	2.66±0.03	1.32
	4-28 (D^4,E^5,D^{12})	0.67±0.12	2.61±0.04	1.29
	8-28	1.97±0.59	2.59±0.07	1.28
	8-28 (D^{12})	1.42±0.57	2.55±0.07	1.26
	18-28	1.02±0.15	3.23±0.05	1.60
B. HMM	1-28	0.46±0.05	0.84±0.01	1.71
	1-24	0.27±0.05	0.73±0.05	1.48
	1-20	0.14±0.03	0.58±0.01	1.18
	1-14	0.12±0.03	0.68±0.01	1.38
	1-7	2.45±0.88	0.52±0.01	1.07
	4-20	0.11±0.02	0.56±0.03	1.14
	8-20	0.15±0.02	0.53±0.02	1.08
	4-28	0.27±0.03	0.73±0.01	1.49
	8-28	0.31±0.08	0.74±0.08	1.51
	18-28	0.33±0.09	0.73±0.01	1.49

[a] At a 1:1 mole ratio of actin to S1, where both proteins had a concentration of 2.5 uM, the initial velocity in the absence of peptide was 2.02 s^{-1}. At a concentration of HMM of 2.5 uM, the initial velocity in the absence of peptide was 0.49 s^{-1}.

[b] The initial activity of acto-S1 or HMM were taken to be V_o.

Unlike the S1 ATPase assay, where actin 1-28 and 4-28 were the only analogs to activate the ATPase activity, all actin analogs activated the acto-S1 ATPase rate. However, the analogs were less effective than actin 1-28 (Figure 34). This supports the conclusion that all residues within actin 1-28 are necessary for full biological function. The deletion of amino acid residues 1-3 from the N-terminus of actin 1-28 resulted in a substantial decrease in V_{max} (1.77 *vs* 1.32, respectively, Table 20). It is only with further deletion of residues 4 to 7 (peptide 8-28) that there was any significant increase in K_{ATPase} (1.97 μM for actin 8-28 *vs* 0.64 μM for actin 4-28). The deletion of amino acid residues 25 to 28 from the C-terminus of actin 1-28 (actin 1-24) resulted in no change in K_{ATPase} but a decrease in V_{max} (1.77 to 1.51). It is interesting that further deletion to residues 1-14 had little effect on K_{ATPase} or V_{max} (compare actin 1-24 and actin 1-14, Table 20), however, deletion of residues 1-7 caused a large decrease in V_{max} (1.11 compared to 1.56 for actin 1-14). In competition with F-actin, the N- and C-terminal regions of 1-28 contribute in a similar fashion for binding and activation of the ATPase activity (for example; actin 1-14 had a K_{ATPase} and V_{max} of 0.59 μM and 1.56 while actin 18-28 had values of 1.02 μM and 1.60). Once again, the central region, residues 8-20 (actin 8-20) affected the ATPase activity (V_{max}, 1.42) and substantially increased K_{ATPase} compared to the effect of actin peptide 1-28 (3.01 μM *vs* 0.46 μM).

THE EFFECT OF ACTIN ANALOGS ON THE HMM ATPase ACTIVITY

All actin analogs activate the HMM ATPase activity but are less effective than actin 1-28 (Figure 35). Again this supports the conclusion that all regions within residues 1-28 are required for full biological activity. Table 20 lists the K_{ATPase} and V_{max} values of the various actin analogs for HMM.

The deletion of amino acid residues 1-3 from the N-terminus of actin 1-28 resulted

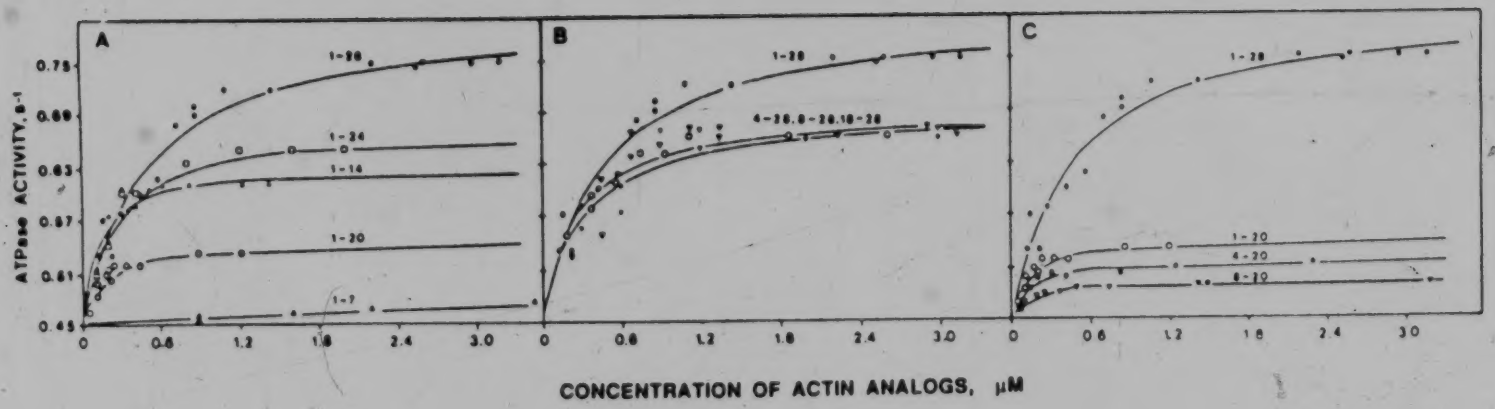

Figure 35. The effect on HMM ATPase activity by C-terminal (panel A), N-terminal (panel B) and both C- and N-terminal truncated (panel C) actin 1-28 analogs. The HMM ATPase rate was 0.49 s^{-1} in the absence of peptide. The concentration of HMM was 2.5 μM. The assay conditions are as described in the Method section. Symbols for the actin peptides are as follows: 1-28 (●), 1-24 (□), 1-20 (○), 1-14 (▲), 1-7 (△), 4-28 (○), 8-28 (▽), 18-28 (▼), 4-20 (■) and 8-20 (▽).

in a decrease in V_{max} (1.71 compared to 1.49 for actin 1-28 and 4-28, respectively). Further deletion of residues from the N-terminus had little effect on either K_{ATPase} or V_{max} (compare actin 4-28, 8-28 and 18-28, Table 20). The deletion of amino acid residues from the C-terminus of actin 1-28 caused a decrease in V_{max} (actin 1-28, 1.71; actin 1-24, 1.48; actin 1-20, 1.18). However, at the same time, there was a decrease in K_{ATPase} from 0.46 μM for actin 1-28 to 0.14 μM for actin 1-20. As seen previously with the acto-S1 ATPase assays, further deletion from the the C-terminus resulted in an increase in V_{max} for actin 1-14 (1.38) compared to actin 1-20 (1.18) in the HMM ATPase assay. The least effective peptide analog was actin 1-7 which caused a large increase in K_{ATPase} (2.45 μM) and a minor effect on the HMM ATPase activity (1.07).

In agreement with the results obtained from the acto-S1 ATPase assays, the N- and C-terminal regions of actin 1-28 contribute in a similar fashion to the binding and activation of the ATPase activity (actin 1-14, 0.12 μM and 1.38 compared to actin 18-28, 0.33 μM and 1.49), while the central region 8-20 is not efficient at activating the ATPase activity (1.08).

DISCUSSION

The purpose of this study is to determine which regions of actin 1-28 are required for full biological activity. Therefore, the effect of the synthetic actin peptide, actin 1-28, and a series of deletion analogs on the S1, acto-S1 and HMM ATPase activities were studied. The actin sequence 1-28 can be divided into three regions; N-terminal, C-terminal and central regions, represented by actin peptides 1-14, 18-28 and 8-20, respectively.

Although it is difficult to explain why some of the actin analogs have different effects on K_{ATPase} and/or V_{max} in the three enzyme systems (S1, acto-S1 and HMM), it is not unexpected. HMM consists of the two globular heads of myosin (S1), where each

polypeptide chain extending from the S1 head interacts to form a two-stranded α-helical coiled-coil (S2). There is no reason to believe that the two globular heads could not show cooperative interactions because of the attachment via the S2 rod-like region. In fact, a significant fraction of HMM is bound to F-actin with only one head in the presence of Mg^{2+}-ADP (Duong and Reisler, 1987). As well, the maximum activity induced by actin 1-28 occurs at 0.5 equivalents of HMM (1 peptide per 2 heads of HMM), suggesting cooperative interactions occur between the two heads (Van Eyk and Hodges, 1991). By comparison, S1, which consists of a single head and binds only one actin monomer (Sutoh, 1983; Chen *et al.*, 1985; Greene, 1984; Heaphy and Tregear, 1984), reaches its maximum activity at approximately 1.5:1 ratio of actin peptide to S1. In the case of acto-S1, S1 is bound to filamentous F-actin and the actin peptide 1-28 or peptide analogs compete with F-actin for S1. Thus, the three enzyme systems are distinctly different from one another. It is known that differences in biological activity between different ATPase assays have been reported. For example, TnC mutants in which the central helix had been altered showed different effects in the acto-S1, and actomyosin ATPase assays (Dobrowolski *et al.*, 1991) as well as in skinned muscle fibre assays (Sheng *et al.*, 1991). Even so, general trends can be seen from the results obtained in the present study using various actin analogs in the S1, acto-S1 and HMM assays.

Most importantly, actin 1-28 is the most effective peptide at activating all three enzyme ATPase activities and, hence, all regions within actin 1-28 are required for maximum biological activity. In addition, the N- and C-terminal and central regions of actin 1-28 have consistent effects in the three assay systems. In the case of S1, deletion of residues 4-7 and 25-28 result in inhibition of the ATPase activity and therefore are important for inducing the conformational change in S1 that is required for activation of the ATPase activity. The central region (represented by actin 8-20) binds poorly to S1 and also inhibits the ATPase activity (Table 19). In the acto-S1 and HMM ATPase assays, deletion

of N-terminal residues cause a progessive decrease in V_{max} (Table 20). Similarly, deletion of C-terminal residues cause a decrease in V_{max}. There are two exceptions, actin 1-14 and 18-28. In both acto-S1 and HMM assays, actin 1-14 induces higher activities than peptides that are longer or shorter than itself, while actin 18-28 has equivalent activity to actin 4-28 and 8-28 in the HMM assay and substantially higher rates in the acto-S1 ATPase assay. These results indicate that deletion analogs comprised of longer or shorter sequences than the exact sequence required for binding to the target protein will be less effective than the analog which contains only the sequence of the exact binding site. In fact in either assay, the N-terminal peptide, actin 1-14, and C-terminal peptide, actin 18-28, have similar effects on binding and activation of the ATPase activity. On the other hand, the central region is not important, since actin 8-20 is poor activator of the acto-S1 and HMM ATPase activities.

The recent three-dimensional structure determined by X-ray crystallography of actin:DNAase complex supports the conclusion that the N- and C-terminal regions of actin 1-28 are the main regions involved in S1 interaction. In the structure of actin, residues 1-7 and 22-28 form surface-exposed loops, while the remaining residues 8-21 are involved in a 5-stranded β-sheet (Kabsch *et al.*, 1990). In fact, residues 1-7 have high temperature coefficients, indicating that these residues are mobile. Since residues 1-7 and 22-28 are located on the surface, this infers that these regions are accessible for interaction with actin-binding proteins -- in this case S1. Because of the involvement of residues 8-21 in the β-sheet, many residues are unavailable to interact with S1 . This concurs with the results obtained for actin peptide 8-20. As well, NMR results on the interaction between actin fragment 1-44 and S1 in the absence of ATP suggested that residues 1-9, F21 or 31 and H40 of actin are involved in S1 binding (Moir and Levine, 1986 and Moir *et al.*, 1987). In addition, the antiprotein antibodies affected by sequence changes at actin residues 2 and 3 (skeletal *vs* cardiac actin) and an antipeptide antibody raised against residues 1-7 partially inhibited the interaction between F-actin and S1 (Méjean *et al.*, 1986, 1987 and Miller *et*

al., 1987), and the antipeptide antibody completely inhibited the S1-induced polymerization of G-actin by blocking the initial binding between the S1 and actin (DasGupta *et al.*, 1990). These results suggest actin residues 1-7 are at, or near, an S1 binding site. On the other hand, no interaction between S1 and a synthetic actin peptide (Y8) 1-8 could be detected by equilibrium dialysis (Miller *et al.*, 1987), suggesting this peptide does not interact or interacts weakly with S1. The results from this study clearly show that the synthetic actin peptide, actin 1-7, binds HMM and S1 under conditions where actin is present (Figures 36 and 37). The importance of residues 1-7 is supported since deletion of amino acid residues 1-7 (peptide 8-28) reduced both the binding and biological activity in the S1, acto-S1 and HMM assays compared to actin 1-28 (Figures 34, 36, and 37). These results indicate the importance of actin residues 1-7 for binding S1 and activation of the ATPase activity. However, since the actin peptide 1-14 is a more effective activator than actin peptide 1-7, residues within amino acid sequence 8-14 enhance binding and potency of this region.

The advantage of this present work is that the bioassays can determine the effect of a deleted region on both the apparent dissociation constant (K_{ATPase}) and biological activity (V_{max}). The previous work using immunological techniques indicated only potential sites of interaction since steric blocking by an antibody can occur without direct involvement of the epitope in the interaction. It must be noted that the NMR and immunological studies were carried out in the absence of ATP, and it has been suggested that the conformation of actin, in particular residues 1-7, is altered depending on whether a nucleotide is bound to S1. When residues 1-7 of F-actin are modified with EDEANS, the modified actin increased the K_M and decreased V_{max} in a S1 ATPase assay compared to native F-actin. In the absence of the ATP (rigor conditions) there was no difference in the interaction of myosin with modified or unmodified actin (Bertrand *et al.*, 1989). This ATP-dependent change in actin residues 1-7 is not unprecedented, since S1 itself undergoes substantial

conformational changes upon interaction with ATP. It is intriguing that when actin is cross-linked to S1, the proteins in the complex appear to have different interactions depending on whether ATP is present or absent (Murphy, 1974; Chen *et al.*, 1985; Arata, 1986; King and Green, 1987). The nucleotide-dependent change of S1 seems to affect all regions of the S1 head (Hiratsuka, 1990a,b), including the area around the reactive thiols, SH1 and SH2 (Seidel and Gergely, 1971; Aquire *et al.*, 1986; Reisler *et al.*, 1974), which are involved in ATP (Lu *et al.*, 1986) and actin binding (Yamaguchi and Sekine, 1966; Suzuki *et al.*, 1987; Eto *et al.*, 1990; Duke *et al.*, 1976). This is significant, since an actin fragment of residues 1-44 perturbed a NMR label located at SH1 of S1 (Moir *et al.*, 1987). This result implies that the N-terminal region of actin (residues 1-44) is situated in close proximity to SH1. It was previously demonstrated S1 is sensitive to conformational changes upon actin peptide binding since the peptide-protein complex aggregated (Van Eyk and Hodges, 1991).

In conclusion, this study demonstrates that actin residues 1-28 are important not only for binding to the S1 head but for activation of the ATPase activity. The three regions of actin residues 1-28 are important for activation of the ATPase activity however the dominant interaction is with the N- and C-terminal regions. The N- and C-terminal regions of actin 1-28 are equally important for the induction of biological activity and both regions produce the most effective and efficient response on the ATPase activity.

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CHAPTER 10

SYNTHETIC PEPTIDE STUDIES ON THE INTERACTION OF THE N-TERMINAL REGION OF ACTIN WITH TROPONIN

INTRODUCTION

Recent studies from numerous laboratories indicates that the N-terminal region of actin is involved not only with S1 but also TnI. The N-terminal region of actin (residues 1-12) was crosslinked (EDC) to TnI (Grabarek and Gergely, 1987). As well, an actin fragment composed of residues 1-44 was shown to interact with a TnI inhibitory fragment, residues 96-116 (Levine *et al.*, 1988). Therefore, the synthetic actin peptide 1-28 and several truncated analogs were used to probe the acto-Tn interaction. It is remarkable that another component of the Ca^{2+}-dependent switch that controls muscle relaxation and contraction seems to involve a switch of the N-terminus of actin between myosin and TnI. Part of this work is published in 'Peptides as Probes in Muscle Research' (Rüegg, J.C, ed) Springer-Verlag, Heidelberg.

RESULTS AND DISCUSSION

INTERACTION BETWEEN ACTIN 1-28 AND Tn

In order to determine whether the actin peptide (1-28) can interact with the Tn complex, high performance size-exclusion chromatography (SEC) was performed. The actin peptide 1-28 bound Tn in the absence, but not in the presence, of Ca^{2+} (Fig. 36). The actin 1-28/troponin complex (peak 1) was easily separated from free peptide (peak 2) by size-exclusion chromatography. Analyses of peak 1 by reversed-phase microbore chromatography (panel C), where all components are separated, clearly shows that the actin peptide is eluted with troponin due to complex formation. In the presence of Ca^{2+}, troponin did not bind actin 1-28, as shown in the analysis of peak 3 by reversed-phase

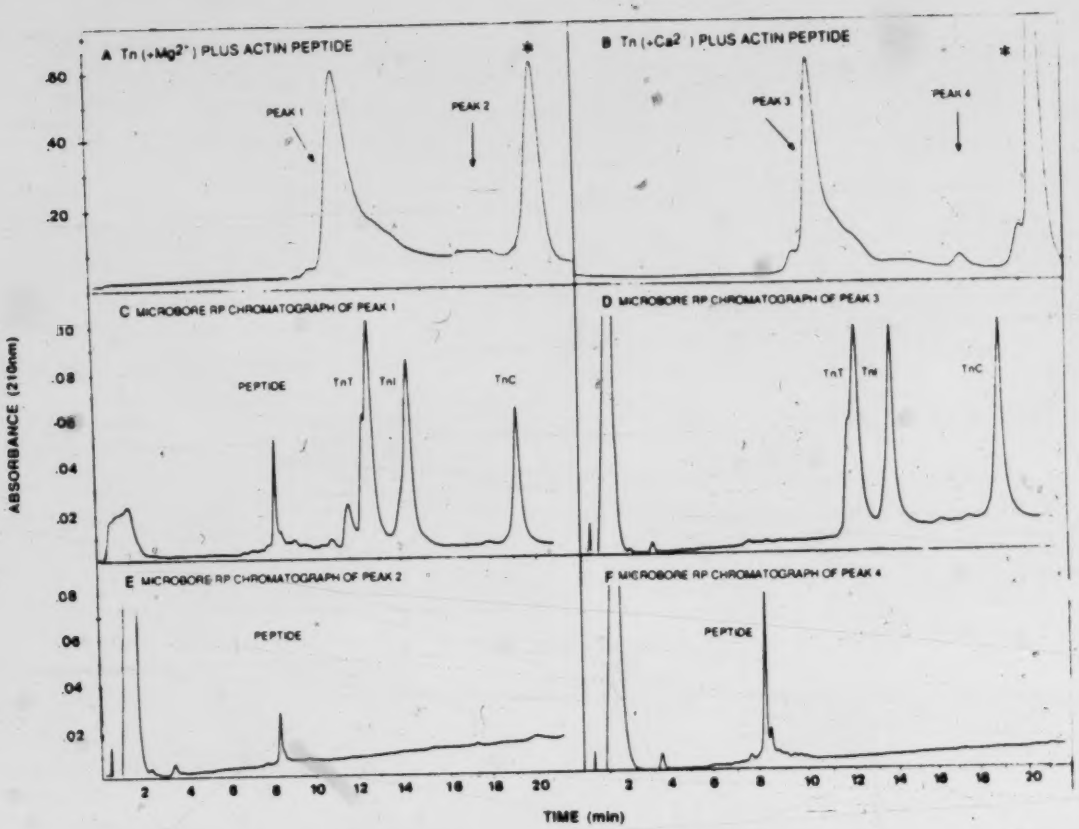

Figure 36. Actin peptide 1-28 and Tn interaction determined by size-exclusion chromatography. Panel A and panel B, respectively, show the elution profile of a mixture of the actin peptide and Tn in the absence of Ca^{2+} (presence of Mg^{2+}) and presence of Ca^{2+}, at a 1:1 mole ratio (3 nmole of each component). The peptide-protein mixtures were run on a high performance size-exclusion column, in a buffer consisting of 10 mM Tris, 100 mM KCl, in the presence of either 5 mM $MgCl_2$ or 2 mM $CaCl_2$, pH 6.8 at a flow rate of 0.5 ml/min. A peak (*) corresponding to the βME that was present in the Tn sample to ensure the cysteine residues of TnI and TnC remained in the reduced state was eluted following the free peptide (panel A and B). The various peaks (1 to 4) were collected and analyzed on a reversed-phase microbore column. A linear AB gradient (2% B/min) (where solvent A is 0.05% aq. TFA and solvent B is 0.05% TFA in acetonitrile at a flow rate of 0.1 ml/min) was used to separate the 3 subunits of Tn from the actin peptide (panels C to F).

chromatography (panel D). Instead, all of the peptide was eluted in peak 4 (compare panels D and F). Furthermore, size exclusion chromatography showed that actin 1-28 was a complex with the TnI subunit of the Tn complex (data not shown). These results suggest that when Mg^{2+} is bound to the high affinity sites of TnC, the conformation of TnI is such

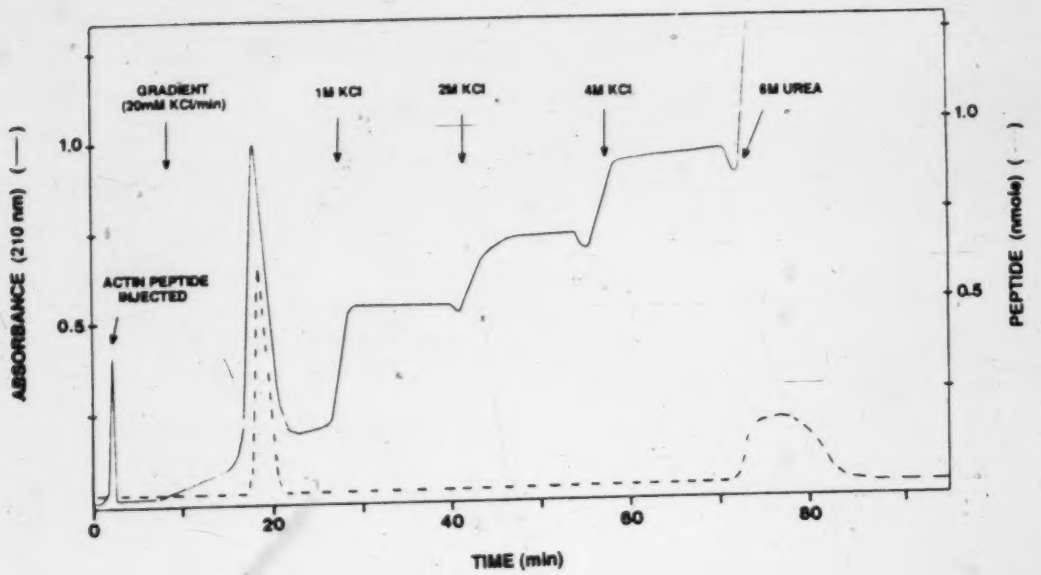

Figure 37. Elution profile of actin peptide 1-28 from a skeletal TnI HPAC column. Actin 1-28 (5 nmoles) was loaded on the column in a buffer consisting of 10 mM Tris, 1 mM DTT, pH 6.8 at 0.5 ml/min. After a 10 minute isocratic delay, a 20 mM KCl/min gradient was run till the KCl concentration was 200 mM. Four different step gradients consisting of loading buffer plus 1 M, 2 M, or 4 M KCl, and finally 6 M urea were applied. One minute fractions were collected and the peptide quantitated by radioactive measurements (- - -) of each fraction.

that actin 1-28 can bind. On the other hand, when Ca^{2+} binds to the low affinity Ca^{2+} binding sites of TnC, the conformation of TnC is altered and this change is transmitted to TnI. The conformation of TnI is affected in such a manner that actin 1-28 can no longer interact. Therefore, the interaction between TnI and actin 1-28 is sensitive to the Ca^{2+} dependent changes in TnC.

INTERACTION BETWEEN ACTIN 1-28 AND TnI

High performance affinity chromatography employing a skeletal TnI derivatized column was used to determine the strength of the interaction between various actin analogs and TnI. The lower the concentration of KCl required to elute an analog from the TnI

HPAC column, the more critical the deleted residues are for the acto-TnI interaction. The actin analogs (1-7, 1-14, 8-20 and 18-28) bound less tightly to the TnI HPAC affinity column than actin 1-28, suggesting that residues 1-28 of actin is the minimum sequence required for the strongest TnI interaction (Table 21). It is surprising that some actin 1-28 (approximately 20-30 % of the total peptide bound to the TnI column) bound so tightly to the TnI column that 6M urea was required to elute the peptide (Fig.37). This was not the case with the other actin analogs. The significance of this extremely tight binding of actin 1-28 to the column is unknown and may be non-specific interaction with the matrix. Low

TABLE 21. RETENTION OF ACTIN ANALOGS DURING TnI AFFINITY CHROMATOGRAPHY[a]

	Elution conditions[b]	
Actin peptide	mM KCl	6M urea
1-28	176	+
1-14	160	-
1-7	95	-
8-20	no binding	-
18-28	85	-

a Analogs were chromatographed on an Ultraffinity column derivatized with skeletal TnI. Peptides were eluted by applying a linear AB gradient of 15 mM/min, where solvent A was 20 mM Tris, 0.1 mM EGTA, 1 mM DTT, pH 6.8 and solvent B was solvent A plus 1.0 M KCl. The column was washed with 6 M urea, 20 mM Tris, pH 6.8. If peptide was eluted under these conditions it is denoted by a plus symbol (+).

b KCl concentration required to elute a peptide was calculated from the retention time minus t_g (gradient delay time) times the gradient rate of 15 mM KCl/min.

derivatization of TnI to the silica matrix could leave a large number of negative charges available for ionic interactions between actin 1-28 and the matrix support. As well, the manner of attachment itself could lead to a small percentage of incorrectly folded TnI such that actin 1-28 could interact with the both types of TnI. Actin 1-14 bound almost as well to TnI as actin 1-28 and considerably more tightly than actin 18-28 (Table 21). This indicates that residues 1-14 are extremely critical for the TnI interaction. The smaller peptide, actin 1-7 bound to TnI but less tightly than actin 1-14, indicating that residues 8-14 enhances the TnI interaction. Since actin 8-20 did not bind at all to TnI then, residues 8-14 are not sufficient for binding but requires residues 1-7 to be present. Therefore, unlike the S1 and HMM interaction, the N-terminal region of 1-28 is more important than the C-terminal region for the interaction between actin and TnI.

Previous work which indicated that the N-terminal region of actin binds to residues 96-116 of TnI (Levine *et al.*, 1988) is confirmed since the synthetic TnI inhibitory peptide, TnI 104-115, successfully competed with TnI (derivatized to the HPAC column) for the actin 1-28 (Fig. 38, panel A). Another TnC-binding peptide, mastoparan was also able to compete with actin 1-28 for the TnI affinity column but was considerably less effective than the TnI peptide (Fig. 38, panel B). Both the TnI peptide and mastoparan are proposed to bind to the same site on TnC (Chapter 4). Therefore, TnI peptide binds to actin residues 1-28 as well as to TnC near helix E. Hence, it is probable that the conformation of actin peptide 1-28 bound to TnI is similar to the conformation of the TnI binding site on TnC.

RECENT DEVELOPMENTS

The structure of actin peptide (1-28) free in solution or bound to either Tn (in the absence of Ca^{2+}) or S1 was recently determined using various NMR techniques (Van Eyk *et al.*, 1991; Söennichsen and Sykes, 1991). The chemical shifts and temperature-dependence of the amide chemical shifts of the peptide in solution are indicative of structure

in the regions D3-T5, G13-L16 and G23-A26. The remaining parts of the peptide, (T^5-A^7, L^8-G^{13}, V^{17} -A^{22}) are flexible and in an extended conformation. Since the NOESY-spectrum lacks any $d_{\alpha\alpha}$ - or side chain contacts and the presence of several sequential side chain contacts (L^8V^9 and $L^{16}V^{17}$) is incompatible with a β-sheet such as the one present in the structure of G-actin determined by X-ray crystalography, a double stranded β-sheet structure is not present in this peptide in solution. This finding is not surprising.

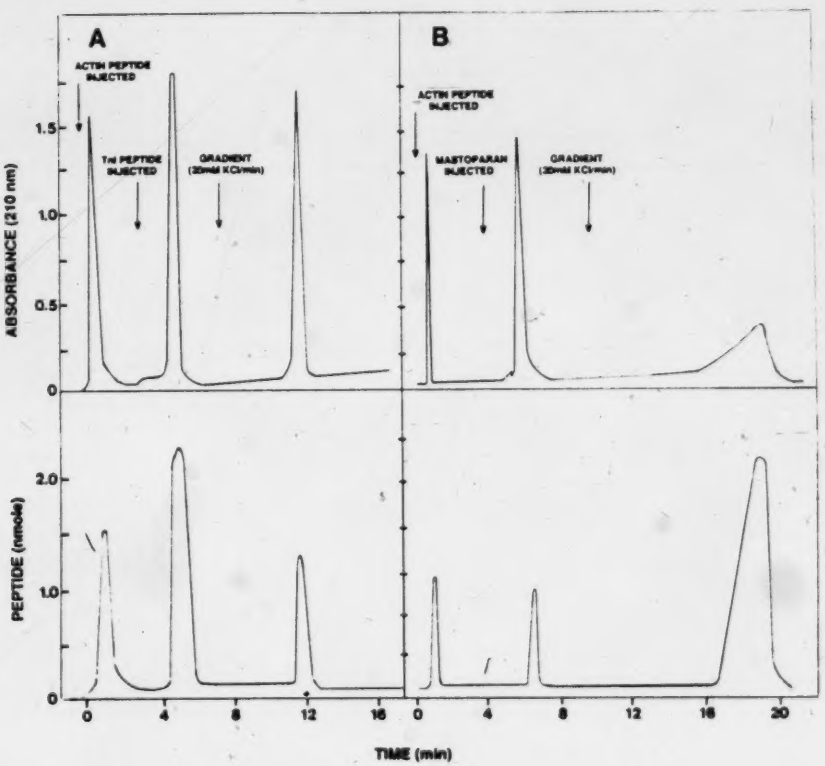

Figure 38. Competition between TnI and the synthetic TnI peptide (104-115) and mastoparan for the actin peptide 1-28. The actin peptide 1-28 (16 nmoles) was applied to the TnI HPAC column using the same conditions as described in figure 37. Either the TnI peptide or mastoparan was then applied to the column isocratically then followed by a linear 20 mM KCl/min gradient. Fractions were collected and the radioactively labelled actin peptide peptide (the only peptide that was labelled) was quantitated by radioactive measurements of each fraction.

since the structure of G-actin, the antiparallel double strand of residues L^8 to F^{21}, is stabilized through further strands on each side, a stabilization which is missing in the case of the shorter peptide.

To obtain structural information about the peptide when bound either to troponin (in absence of Ca^{2+}) or myosin, transferred NOESY (trNOESY) experiments were performed. There was minimum change in crosspeak intensity in the region between G^{13} and G^{20}, reflecting the same conformation in all three spectra, i.e., free in solution and bound to either troponin or S1. Therefore, this region is either not involved in binding or not affected by it. The largest change occurred in the N-terminal part of the peptide, in particular between D^1 and A^7, suggesting that this is the major site of interaction. These results suggest the formation of a helix in this part of the peptide; however no medium range ($d\alpha N(i,i+3)$) connectivities were observed. Therefore, it seems that there is a transition from an extended peptide structure towards a helix such that there is a series of turns or a loose, distorted helix. Interestingly this region (residues 1-7) shows identical nOe-patterns when the peptide is bound to either troponin or S1.

Differences between the free and bound form, as well as between the two bound structures, were detected in the regions D^{11}-N^{12} and F^{21}-R^{28}. The latter region forms a second binding site, which is structured differently depending on whether the peptide is bound to S1 or TnI. Since the binding of the N-terminal part of the peptide to both proteins is identical, the conformational differences in this region (F^{21}-R^{28}) may reflect the differences on a structural level between the binding of the peptide with troponin and S1.

The regions of structure of the peptide when bound to Tn or S1 can be correlated to the regions of actin 1-28 that are critical for binding and biological activity (work presented in Chapters 9 and 10). Actin 1-28 can be divided into three distinct regions. The central region, (represented by actin analog 8-20) has minimal contact with S1 and is not efficient

at activating the acto-S1 or HMM ATPase activity. As well, the peptide does not bind to TnI. This fact is consistent with the finding that the conformation of the central residues 8-20 (with one exception) is not affected by binding to either troponin or S1 which indicates that the central region does not bind or has only minimal contact with the proteins. The N-terminal region of actin 1-28 (residues 1-7) strongly interacts with troponin I and myosin. For example, actin analog 1-14 binds tightly to a TnI affinity column and is effective at activating the S1 and HMM ATPase activity. This strong interaction of the N-terminus of actin 1-28 with TnI and S1 induces a conformational change, especially in the first 7 residues of actin 1-28. It is interesting that the conformation of the actin peptide is similar when it is bound to either troponin or S1. This implies that the actin-binding site of TnI and S1 for the N-terminal residues of actin are similar.

The C-terminal region of actin 1-28 (residues 20-28 and represented by actin analog 18-28) also binds to both proteins. In the case of the interaction between actin 1-28 and S1 or HMM, the C-terminal interaction is as important as the binding of the N-terminus, while it is considerably less important when actin 1-28 binds to troponin. This difference can be observed at the structural level. While binding of the peptide to either protein induces a change in the conformation of residues 21-28, thus proving the direct involvement of these residues in binding, the conformation of this region in the complex with either protein is not the same. This difference in conformation may reflect differences of the actin binding sites of TnI and myosin and the ability of actin 1-28 to adapt and fit these two proteins.

Another small difference between the two bound conformations was detected at residues D^{11}-N^{12}. The significance of this change is, so far, unclear. Since the change was limited to these two residues, it seems unlikely that this change is due to peptide-protein interaction in this region. Therefore, this conformational change may be due to differences in the binding of the two different target proteins which are reflected by small differences in the protein conformation in the central region of the peptide. In the intact

protein (crystal structure), these residues are buried in the core of the protein as part of the 5-stranded β-sheet, and would be limited in the amount of movement possible. Still, it is possible that D^{11}-N^{12} serves as a hinge region meeting different distance requirements between the N-terminal and the C-terminal binding sites of the peptide when bound either to TnI or S1. For the intact protein, this would imply that binding to troponin or S1 leads not only to a conformational change at the N and C-terminal binding sites, but also alters the central β-sheet structure. This would affect the conformation of the whole subdomain.

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CHAPTER 11

DISCUSSION

The use of synthetic peptides which mimic functionally important regions of TnI and actin has greatly increased our understanding of the regulatory mechanisms involved in muscle contraction. The studies presented in this thesis have clearly demonstrated that actin peptide 1-28 and skeletal TnI inhibitory peptide 104-115 (and analogous cardiac TnI peptide 137-148) represent regions of their native proteins that are critical for regulation of muscle contraction. The native synthetic peptides each interact with two different target proteins: actin peptide 1-28 can bind TnI and various myosin fragments while the TnI peptide 104-115 can interact with TM-actin or TnC. This suggests that in muscle regulation a flip-flop mechanism occurs in which the N-terminal region of actin and the inhibitory region of TnI switches between binding sites on their various target proteins. As well, actin peptide 1-28 binds Tn, in particular TnI only in the absence of saturating Ca^{2+}, while the inhibition of the S1 ATPase activity induced by TnI peptide is released by TnC in the presence of saturating Ca^{2+}. Therefore, the interactions between the TnI inhibitory region and the N-terminus of actin and their target proteins is Ca^{2+} dependent .

EXPANDED MODEL OF MUSCLE REGULATION

The current model for muscle regulation proposes that in the presence of saturating Ca^{2+} (muscle contraction), movement of TnI-TM on the actin filament alters the actin interaction with myosin, resulting in activation of the ATPase activity due to increased release of Pi from the myosin S1 head. In the absence of saturating Ca^{2+} (muscle relaxation), movement of TnI-TM on the actin filament alters the conformation of actin so that the interaction between actin and S1 is minimized, thus reducing the ATPase activity. In the expanded model of muscle regulation based on the information obtained from using

the synthetic peptides of actin and TnI, control of the TnI-TM position resides in the fact that the TnI inhibitory region can flip-flop between TnC and actin-TM. Similarly, the N-terminal region of actin (residues 1-28) acts as a chemical switch, flip-flopping between a binding site on TnI, in particular the inhibitory region of TnI, and a binding site on S1. The particular interaction which dominates in the absence or presence of Ca^{2+} is dependent on the relative binding affinities of the TnI inhibitory region and its binding site on TnC and TM-actin, and the N-terminal region of actin and its binding sites on TnI and S1. Since the interaction between actin-TnI and TnI-TnC is dependent on whether Ca^{2+} is bound to regulatory low affinity Ca^{2+} binding sites of TnC, the rate of ATP hydrolysis by myosin can be directly linked to Ca^{2+} binding to TnC.

In the absence of Ca^{2+}, when either Mg^{2+} or Ca^{2+} is bound to the high affinity sites of TnC, the interaction between TnI and actin (plus-TM) is dominant (Fig. 39). Thus, muscle relaxation is promoted due to inhibition of myosin ATPase activity (Van Eyk and Hodges, 1986). In contrast, when Ca^{2+} binds to the regulatory low affinity Ca^{2+} binding sites of TnC, TnI inhibition is released since TnI residues 104-115 has a stronger binding affinity for TnC in the presence than in the absence of saturating Ca^{2+} (Van Eyk and Hodges, 1988) and actin residues 1-28 cannot bind to Tn in the presence of Ca^{2+} (Van Eyk and Hodges, 1991a). Hence, actin residues 1-28 then interact with the myosin S1 head causing potentiation of the ATPase activity by altering the conformation of the S1 head such that release of Pi occurs, thus promoting muscle contraction.

The interactions described in Fig. 39 do not take into account the important role of TM in propagating the biological activity of the TnI and actin interactions. It is well documented that the presence of TM in the ATPase assay greatly enhances the activity of TnI and TnI peptide 104-115 (Talbot and Hodges, 1979, Eaton *et al.*, 1975) through multi-site interaction of a single TM-Tn unit with up to 7 monomers in filamentous actin. TM-Tn which occupies a several positions on the actin filament is controlled by Ca^{2+} binding

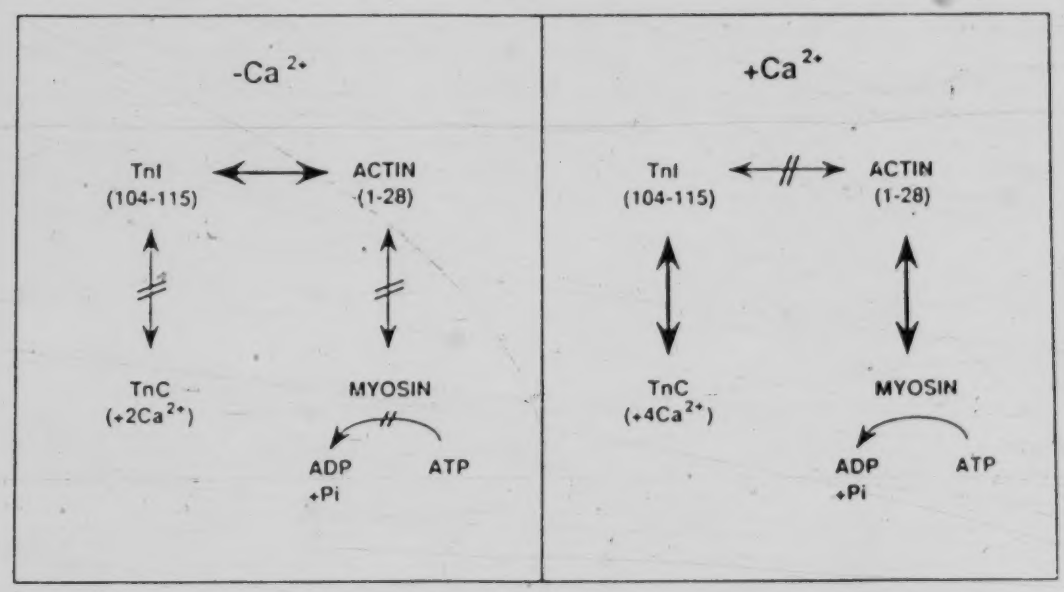


Figure 39. Schematic showing the dominant protein interactions involved in the regulation of muscle contraction. $-Ca^{2+}$ denotes when the high affinity Ca^{2+} binding sites of TnC are filled, conditions where muscle relaxation occurs, while $+Ca^{2+}$ denotes when the high and regulatory low affinity Ca^{2+} binding sites are filled and hence muscle contraction takes place .

to TnC and the nucleotide bound to S1 head (Hill, 1983, Hill *et al.*, 1984). Therefore, the extent of blocking of the acto-S1 interaction by TM-TnI is dictated by the conformation of the myosin head and, in addition, by the conformation of the proteins within the thin filament which is dependent on whether the regulatory low affinity Ca^{2+} binding sites of TnC are filled (Fig. 40). Therefore, the conformational changes associated with the

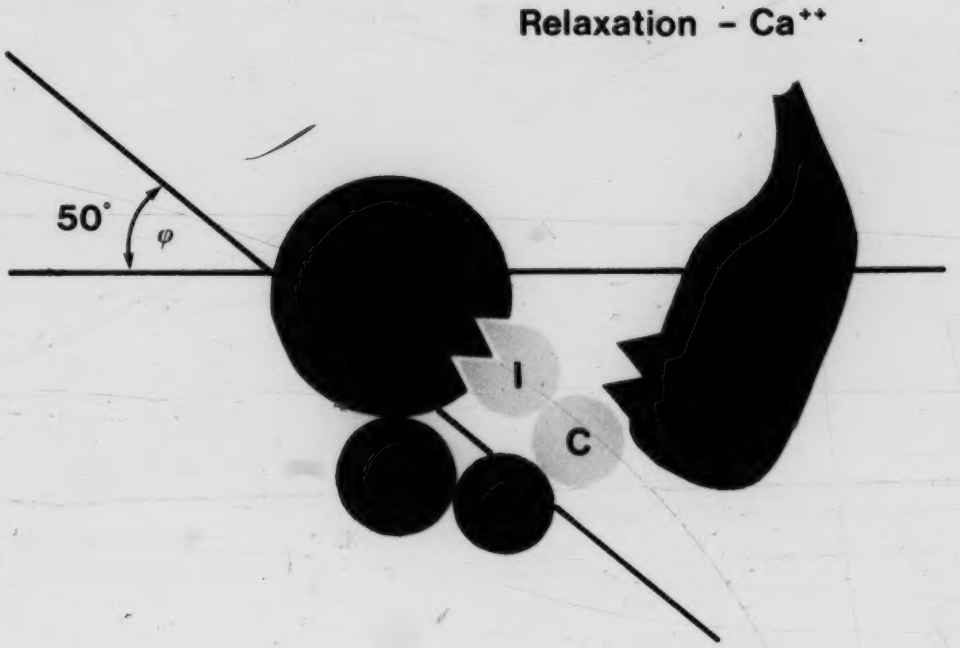
binding of actin to myosin (formation of a crossbridge) and the changes in TnC upon Ca^{2+} binding influence one another. The nature of the actin-dependent conformational changes in S1 head which stimulates the ATPase activity is not clear. On the other hand, there is considerable knowledge about the Ca^{2+}-dependent conformational changes that occur in TnC and TnC-TnI.

MOLECULAR EVENTS THAT CONTROL MUSCLE CONTRACTION

TRANSMISSION OF THE Ca^{2+} SIGNAL VIA TnI

The signal that initiates muscle contraction causes Ca^{2+}-dependent conformational changes in TnC which alter the conformation of the other proteins in the Tn complex,TnI and TnT. This in turn influences the conformation and position of TM on the actin filament which finally alters the actin-S1 interaction. The synthetic peptides of TnI and actin are very useful in probing the molecular events involved in the transmission of the Ca^{2+} signal from TnC to the ATP active site on S1.

In a manner similar to its native protein, the binding of the TnI peptide to TnC increases the Ca^{2+} affinity of TnC (Chapter 4, Van Eyk & Hodges, 1987; Chapter 5, Van Eyk & Hodges, 1988; and Chapter 7, Van Eyk *et al.*, 1991b). It is significant that the TnI peptide interaction with TnC causes an increase in the Ca^{2+} affinity of the regulatory low affinity sites I and II of skeletal TnC (located in the N-terminal domain) with only minor effects on the high affinity binding sites III and IV (located in the C-terminal domain, Chapter 7). In addition, binding Ca^{2+} to the regulatory sites of TnC increases the strength of the interaction between the TnI peptide and TnC since a higher concentration of KCl is required to elute the TnI peptide from the TnC affinity column in the presence of Ca^{2+} than in the presence of Mg^{2+} (Chapter 5). Therefore, the interaction between the TnI peptide and TnC increases the Ca^{2+} affinity of the low affinity sites of TnC, while Ca^{2+} binding to these sites (compare Ca^{2+} to Mg^{2+}) increases the TnI peptide affinity for TnC.


Relaxation - Ca++
50°
φ
I
C

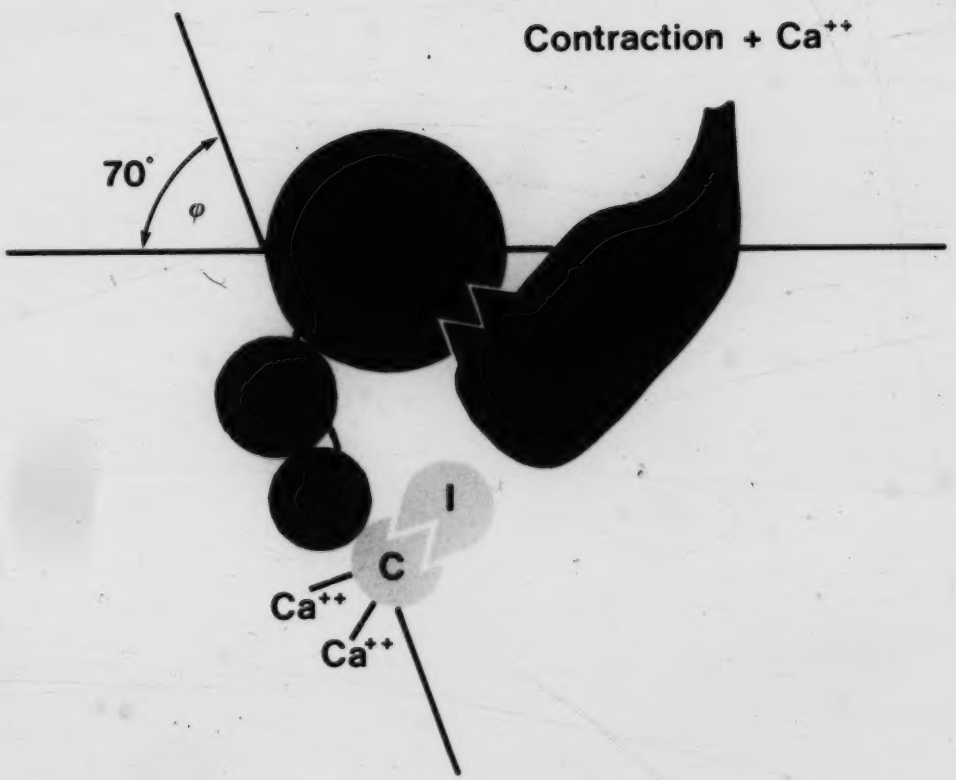

Contraction + Ca++
70°
φ
I
C
Ca++
Ca++

Figure 40: Cross section of skeletal muscle thin and thick filaments and the possible changes in protein-protein interactions upon Ca^{2+} binding. The relative positions of actin, tropomyosin (TM), Tn (I, T and C) and the head of the myosin molecule (S1) move with respect to one another depending on the concentration of Ca^{2+}. In the absence of Ca^{2+}, the TnI inhibitory region (hatched area) interacts with the N-terminal region of actin (black area) causing relaxation of the muscle. In the presence of Ca^{2+}, the inhibitory region of TnI interacts with TnC (most likely residues 89-100) and the N-terminal region of actin interacts with the myosin head causing activation of the ATPase activity and hence contraction of the muscle.

The difference between the skeletal and cardiac TnI inhibitory sequences is a single amino acid substitution of Pro to Thr at position 110 of the skeletal sequence. The cardiac inhibitory peptide 137-148 inhibits force development in skinned muscle fibres but is less efficient than the skeletal TnI peptide (Rüegg *et al.*, 1991c; Chapter 6, Rüegg *et al.*, 1989). Each of the two native TnI peptides affected Ca^{2+} binding to the low affinity sites of TnC. However, there are differences in the direction and magnitude of the pCa shifts (Chapter 7). The importance of this single amino acid is further demonstrated by the difference in the biological action of an analog in which a glycine is substituted at position 110 of the skeletal TnI sequence (Gly 110). Gly 110 inhibits the acto-S1 ATPase activity but is considerably less effective than the skeletal TnI peptide (Chapter 5). Unlike the skeletal TnI peptide, Gly 110 does not alter force development in skinned muscle fibres (Rüegg *et al.*, 1991). The difference in the action of Gly 110 in the skinned fibre assay compared to the native TnI peptides may be due to a difference in the interaction between the analog and TnC . Even though this analog binds TnC almost as well as the native skeletal peptide (Chapter 5), Gly 110 has a very different effect on the Ca^{2+} binding affinity (Chapter 7). The skeletal and cardiac TnI peptides alter the regulatory low affinity Ca^{2+} binding sites of TnC while the Gly 110 analog alters Ca^{2+} binding at the high affinity sites III and IV . This indicates that the biological activity of the TnI peptides is very dependent on which amino acid is present at position 110 of the skeletal sequence.

Single glycine substituted analogs of the skeletal TnI peptide 104-115 were analyzed in order to determine which amino acid residues are essential for biological activity of this region. All the amino acids were important for binding the peptide to TnC and for the inhibition of the S1 ATPase activity. However, the C-terminal amino acid residues are the most critical residues (Chapter 5). These results indicate that the Ca^{2+}-dependent switch involving the TnI inhibitory region involves specific interaction of the C-terminal residues within the sequence 104-115 which can bind either to TM-actin or TnC, depending on the presence or absence of Ca^{2+}. The NMR-derived structure of the TnI peptide bound to TnC in the presence of Ca^{2+}(Campbell and Sykes, 1991) shows the residues most critical for biological activity are clustered in a small area of the peptide. The critical residues include the three hydrophobic residues which form the hydrophobic pocket of the peptide.

TRANSMISSION OF THE Ca^{2+} SIGNAL VIA ACTIN TO S1

In the absence of Ca^{2+}, TnI residues 104-115 interact with actin residues 1-28. The conformational changes in TnI which occur due to this interaction are not known at the present time. However, the structures of the actin peptide 1-28 bound to both Tn and S1 have been determined and are remarkably similar (Van Eyk *et al.*, 1991a). This similarity suggests that a sequence of amino acids capable of binding different proteins is able to adapt, to some degree, to the various binding interfaces. Hence, the binding interfaces of the various target proteins must be similar to one another such that this common peptide displays only small differences in its structure when bound to either target protein. Therefore, one can speculate that the TnI binding sites on actin and TnC are similar, especially since the C-terminal residues of TnI peptide are critical for binding to both proteins. The TnI peptide primary binding site is located near or at the helix E of TnC and upon binding increases the α-helical content of TnC (Chapters 6 and 7). Similarly, the

binding of TnI to actin 1-28 induces a conformational change from an extended structure in solution to a helix-like conformation between actin residues 1-7. Residues 1-7 are critical for binding of the actin peptide to TnI and S1 (Chapters 9, Van Eyk and Hodges, 1991a; and Chapter 10, Van Eyk *et al.*, 1991a). The binding of S1 to actin 1-28 also induced this helix-like conformation in the first 7 residues of the actin peptide. Therefore, it is possible that binding site for TnI residues 104-115 would be located at or near actin residues 1-7 . In addition, the binding of the TnI inhibitory region could possibly induce the initial seven residues of actin into a helix-like structure.

These regions of TnI and actin are highly charged since TnI residues 104-115 contain 6 positive charged amino acids while the first four N-terminal residues of actin are negatively charged. This suggests that coulombic interactions would play a major role in this interaction. If the binding site on S1 for actin residues 1-7 reflects the binding site on actin for TnI residues 104-115, then by analogy the S1 binding site should be positively charged and mimic the structure of TnI peptide when bound to TnC. In other words, the amino acid side chains that are critical for the interaction between TnI 104-115 and actin 1-7 should be able to be superimpose on the amino acid side chains that are critical for the actin and S1 interaction .

The interaction between actin 1-28 and S1 activates the ATPase activity (Chapter 8) indicating that the conformational change induced by peptide binding to S1 is responsible for the increased in the rate of Pi release and hence an increase in the ATP hydrolysis rate. At the present time, the exact binding site of actin 1-28 on S1 is unknown. There are 3 regions of S1(A1) known to interact with actin (Table 2). The S site located near SH1 and SH2 in the 20 kDa fragment of S1 and the J site located at the 20 and 50 kDa junction are assumed to determine the high affinity of the acto-S1 rigor state and the low affinity site in the absence and presence of ATP, respectively. The activation of S1 ATPase activity by F-actin is assumed to be induced by a transient attachment of the S site with F-actin after

release of the Pi from the nucleotide binding site on S1, while the J site keeps acto-S1 at a steady state of ATPase (Katoh and Morita, 1984). Therefore, binding of the S site to F-actin is necessary for the activation but does not affect the affinity between myosin and actin (Suzuki et al., 1990). During the ATP hydrolysis step, the S site is dissociated from actin but the J site remains bound and hence is responsible for the weak binding states. Therefore, the S site interaction could play a possible role in the Ca^{2+} regulation. Chalovich and Eisenberg (1982) reported that the inhibition of the actin activated ATPase caused by TM-Tn in the absence of Ca^{2+} was mainly due to a decrease in the rate of the kinetic step, and not due to a decrease in affinity between myosin and actin. Furthermore, they showed that TM-Tn inhibited the binding between myosin and F-actin in the strong binding state (rigor or myosin-ADP) but not in the weak binding state. Therefore, TM-Tn in the absence of Ca^{2+} inhibits the binding of F-actin at the S-site such as to cover the site on actin, resulting in inhibition of the release of ATP hydrolysis products. Hence, muscle contraction may be controlled by the Ca^{2+}-dependent interaction between actin 1-28 and Tn which limits the interaction between actin 1-28 and the S site on S1. In the absence of Ca^{2+}, actin residues 1-28 would interact with TnI inhibitory region (residues 104-115). Whereas in the presence of Ca^{2+}, the TnI inhibitory region would interact with TnC, thus allowing actin 1-28 to interact with S site of S1. The interaction between actin 1-28 and S1 would cause an increase in the release of Pi and hence an increase in the ATP hydrolysis rate.

FUTURE INVESTIGATIONS

Though significant information has been obtained from the use of synthetic peptides of actin and TnI, there still remain gaps in our understanding of how, at the molecular level the Ca^{2+}-dependent conformational changes alter the binding interfaces of the muscle proteins, thus changing the binding affinities so that different protein-protein interactions are favoured in the absence or the presence of Ca^{2+}. The structure of the TnI peptide

bound to TM-actin is essential to allow comparison of the structure of the peptide bound to TnC. This will give a clue to how the TnI inhibitory region is able to adapt to fit the two binding sites. As well, work is in progress to analyze single glycine analogs of the cardiac TnI peptide, and to determine the exact amino acids required for interaction with cardiac TnC, inhibition of the acto-S1 ATPase activity and the effect on skinned muscle fibres. Such work will help in understanding how a single residue change in both the cardiac and the skeletal TnI inhibitory regions can alter the biological activity of these different muscle types. Research has just begun using the actin peptide as a probe to understand muscle regulation. First, the amino acid residues that are critical for binding to TnI and activation of S1 ATPase must be determined by using single glycine or alanine-substituted analogs in a manner similar to that done for the skeletal TnI inhibitory peptide. Identification of the myosin binding site is essential and can be achieved by crosslinking of the photoaffinity-labelled actin peptide to S1. As well, the effect of ATP, ADP and Pi on the actin-S1 interaction should provide information on how crossbridge cycling is affected by actin binding. It is clear that the synthetic peptide approach has been able to answer many questions about muscle regulation and in the future promises to be just as fruitful in determining the exact mechanism involved in the complex regulation of muscle contraction.

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